Bot.
2

Volume 32 FEBRUARY, 1952 No. 2

Due March 21
IN STATION LIBRARY
26 1952

SCIENTIFIC AGRICULTURE

AGRICULTURE EXPERIMENT STATION LIBRARY
MAR 7 1952
PURDUE UNIVERSITY

CONTENTS

Tomato breeding for resistance to *Phytophthora infestans* (Mont.) de By. 1. Method of inoculation and preliminary results - *W. Ferguson, L. H. Lyall and H. N. Racicot* 57

Studies in red clover seed production *R. M. MacVicar, E. Braun, D. R. Gibson and C. A. Jamieson* 67

A determinative study of amylolytic, stenothermophilic bacteria isolated from soil - - - *Egon Stark and P. A. Tetrault* 81

Narcissus bulb fly control with methyl bromide fumigation and its effect on the flower production of greenhouse-grown narcissus bulbs - - - *H. Andison and W. T. Cram* 93

Studies on the holocellulose fraction of hardwood leaves *B. B. Coldwell and W. A. DeLong* 99

Air purification and soluble pectin accumulations in Lawfam apples - - - - *P. A. Poapst and W. R. Phillips* 109

Infection of basal shoots of hop plants by *Rhizoctonia solani* *Walter Jones* 114

Selected List of Accessions, Main Library, Department of Agriculture

EDITED BY THE
AGRICULTURAL INSTITUTE OF CANADA
OTTAWA, CANADA

SCIENTIFIC AGRICULTURE

EDITED BY THE

AGRICULTURAL INSTITUTE OF CANADA

OTTAWA, CANADA

EDITORIAL BOARD

R. D. RAMSAY, General Secretary, Agricultural Institute of Canada, Ottawa, Canada—*Chairman.*

J. F. BOOTH, Chief, Economics Division, Marketing Service, Canada Department of Agriculture, Ottawa, Canada.

R. H. COMMON, Professor and Chairman, Department of Chemistry, Macdonald College, Que.

W. H. COOK, Director, Division of Applied Biology, National Research Council, Ottawa, Canada.

E. W. CRAMPTON, Professor of Animal Nutrition, Macdonald College, Que.

T. JOHNSON, Plant Pathologist, Dominion Laboratory of Plant Pathology, Winnipeg, Man.

A. G. McCALLA, Dean, Faculty of Agriculture, University of Alberta, Edmonton, Alta.

G. N. RUHNKE, Research Director, Ontario Department of Agriculture, c/o O.A.C., Guelph, Ont.

H. L. TRUEMAN, Director, Administrative Services, Canada Department of Agriculture, Ottawa, Canada.

J. C. WOODWARD, Chief, Division of Chemistry, Science Service, Canada Department of Agriculture, Ottawa, Canada.

Managing Editor - - - - RUPERT D. RAMSAY
Editor - - - - HILDA GRAY

Published monthly by the authority

of the Chairman of the

National Committee on Agricultural Services

CORRESPONDENCE AND SUBSCRIPTIONS

Address all correspondence to the Agricultural Institute of Canada, Grindley Hall, 338 Somerset Street West, Ottawa, Canada. *Scientific Agriculture* will be mailed to any address in Canada, the United States, Mexico, Central and South America and the British Commonwealth for $3.00 per year; other countries $4.00 per year.

Authorized as second class mail, Post Office Department, Ottawa.

SCIENTIFIC AGRICULTURE

VOLUME 32 FEBRUARY, 1952 No. 2

TOMATO BREEDING FOR RESISTANCE TO *PHYTOPHTHORA INFESTANS* (MONT.) DE BY.

I. METHOD OF INOCULATION AND PRELIMINARY RESULTS[1]

W. FERGUSON,[2] L. H. LYALL[2], AND H. N. RACICOT[3]

Science Service, Department of Agriculture, Ottawa, Canada

[Received for publication June 7, 1951]

The tomato crop is an important one in Canada. In 1948, some 68,340 acres were planted. Ontario accounted for most of this, with 52,040 acres, having a crop value of over $12,000,000. The late blight disease caused by *phytophthora infestans* is a serious annual threat. In Ontario, there is an extensive late blight warning service set up by the Provincial Department of Agriculture to warn growers of outbreaks of this disease and advise preventive measures. The seriousness of the disease makes breeding for resistance an economically important line of research.

LITERATURE REVIEW

According to Boswell and Bostelmann (5) and Luckwill (10) the cultivated tomato originated as wild forms in the Peru-Ecuador-Bolivia area of the Andes. It is believed the cultivated species was carried into Central America and Mexico by Indian migrations although it seems to have been of little importance as an item of food among the Indians until after the arrival of the white man. There is no early evidence of its use among the North American Indians.

The tomato was introduced into Italy about 1550 and soon after spread to other countries of Europe. At first it was regarded as a novelty but by 1750 was grown extensively for food, at least in Italy and Spain. It was brought from Europe to North America and was used as a food plant about 1812 in New Orleans and some 25 years later was grown in the north-eastern States and, presumably, in Canada.

The tomato is a member of the family *Solanaceae*, as is the potato, and for a time was included in the same genus, *Solanum*. It is now a member of the genus *Lycopersicon*, of which there are several species. *Lycopersicon esculentum* contains the horticultural varieties. Muller (13) in his botanical classification of the *Lycopersicons* places the red or yellow fruited species *L. esculentum* and *L. Pimpinellifolium* in the sub-genus *Eulycopersicon*. In the other sub-genus *Eriopersicon* he places those with green, white or purplish fruit and which are usually hairy at maturity. These consist of *L. cheesmanii*, *L. peruvianum*, *L. hirsutum* and *L. glandulosum*. Luckwill (11) in a later classification adheres closely to this but includes an extra species under the sub-genus *Eriopersicon* namely *L. pissisi* which Muller appears to have included in *L. cheesmanii*.

[1] Contribution No. 741 from the Division of Horticulture, Experimental Farms Service, in co-operation with the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa.
[2] Senior Horticulturist and Horticulturist, respectively, Division of Horticulture.
[3] Plant Pathologist, Division of Botany and Plant Pathology.

The causal organism of late blight is the fungus *Phytophthora infestans* which also causes late blight of potatoes. This is not surprising, since both hosts are solanaceous plants. In addition it is believed to have had its origin along with the tomato and potato in the Northern Andes and like them to have spread from there. Naturally the early history of the discovery of this fungus and its ravages is associated with its relationship to the potato.

According to Heald (8) late blight of potatoes was introduced into Europe and North America between 1830-1840. In 1845 it reached epidemic proportions on both continents and was the main cause of the Irish famine of that date. In later years the disease spread to India, Australia and South Africa. In all likelihood it spread on to tomatoes where they were grown at the same time. The organism was finally established as the cause of the disease, by de Bary in Germany, in 1861.

The disease has been well described by numerous workers including Strong (18), Doolittle (6), Berkeley and Richardson (4), and Porter and MacGillivray (16). The leaf symptoms are similar to those on potatoes appearing as greenish-black, water-soaked patches on the older leaves. The spots enlarge rapidly and sometimes show a white, downy growth of the fungus on the lower leaves in moist weather. The stems may also show similar brown areas. The downy growth produces spores which spread the disease to other plants and in moderate wet weather the infection will spread very rapidly. When the fruit become infected a rot develops, usually near the stem end, and spreads over the surface of the fruit.

Breeding for disease resistance has been carried on by numerous workers and against various diseases. Young and MacArthur (19) present some useful information on the horticultural characters of tomatoes. Their work includes an index of 50 characters designated by gene symbols and some 70 characters not designated by gene symbols. Among these, immunity and resistance to *Cladosporium fulvum*, resistance to Alternaria collar rot, Septoria and immunity to Fusarium wilt are designated by gene symbol. There is no information concerning any character which may give resistance to late blight. Porte and Wellman (14) use *L. pimpinellifolium* as a source of resistance to Fusarium wilt in crosses with Marglobe and by selection and backcrosses developed a satisfactory wilt-resistant variety. In their tests for disease resistance various methods of inoculating the soil were used. Satisfactory greenhouse inoculations were made by dipping the roots of the tomato seedlings in a filtered suspension of fungus and spores before planting them in benches of sterilized soil.

Locke (9) found *L. hirsutum* resistant to both Septoria blight and early blight and first generation hybrids of *L. esculentum* × *L. hirsutum* were resistant to septoria blight but only intermediate in resistance to early blight. Moore and Reynard (12) found no varieties with complete resistance to the stem lesion phase of early blight, *Alternaria solani*. Andrus and Reynard (3) used a greenhouse inoculation procedure similar to that described by Porte and Wellman (14) but in this case the leaves of the young plants were dipped in the inoculum. They found no complete resistance to early blight in either horticultural varieties of *L. esculentum* or the species *L. pimpinellifolium* or *L. peruvianum*. Alexander and Wright (1) in their survey of the genus *Lycopersicon* for resistance to tomato

diseases in Ohio and Indiana tested 448 tomato introductions. Some resistance, although not complete, was found for early blight chiefly in *L. peruvianum* and *L. hirsutum*. No observations were recorded for resistance to late blight.

Richards and Barratt (17) conducted a partial survey of the genus *Lycopersicon* for resistance to *Phytophthora infestans*. Inoculations were made by spraying the growing plants with a spore suspension. Tests were made on 137 lots of plants consisting of a varying number of selections of different species including *L. hirsutum*, *L. pimpinellifolium*, *L. peruvianum* and various botanical and horticultural varieties and crosses of *L. esculentum*. They concluded that slight differences in resistance to *P. infestans* may exist within the genus *Lycopersicon* particularly in the field when conditions are not always ideal for infection; but of the plants tested none was found to be highly resistant or immune to the late blight pathogen.

During the severe late blight epidemic in 1946 Andrus (2) made field observations on 44 varieties and listed 22 as susceptible, 5 with some resistance and 17 resistant. It is felt these last might better be classed as free of late blight since they include a number of varieties that are not fully resistant although some are apparently less susceptible than others. Epps (7) reported some resistance to late blight from crosses between *L. esculentum* and *L. pimpinellifolium*, but no resistance was observed in the fruit.

Porte and Walker (15) crossed a strong growing strain of *L. peruvianum* with varieties of *L. esculentum* in a search for resistance to several foliage diseases. The only successful cross was obtained with Prince Borghese, an Italian paste type, and the plants only set fruit when treated with a growth-regulating substance such as naphthalene acetamide or indolebutyric acid, but the fruit were seedless. A few small fruits were obtained from some field grown plants started from cuttings and contained a few seeds. Several crosses were made between the plants produced from these seeds and several varieties of *L. esculentum*. The progeny showed wide segregation. Some steps in the procedure may be of value in a tomato breeding program.

MATERIALS AND METHODS

Experimental work on the problem of finding resistance to *P. infestans* was started in the Division of Horticulture, Central Experimental Farm, Ottawa, in 1948 in co-operation with the Division of Botany and Plant Pathology, Science Service.

Initial work on a limited scale was carried on in the greenhouses in the spring of 1948 and was designed to test the virulence of the isolate of *P. infestans* then available and to try out a proposed technique of inoculation and examination. The tomato variety, Stokesdale, known to be very susceptible in the field, was very heavily damaged under conditions of artificial infection in the greenhouse. It was decided, therefore, to use this variety as a check or control in future testing.

In subsequent tests a standard procedure was followed. The actual testing of strains of tomatoes is carried out in the greenhouses of the Division of Horticulture with inoculations and examinations being made by the co-operating Plant Pathologist. All seed is treated with Arasan

before planting and is sown in steam-sterilized soil. One hundred young seedlings of each strain under test are pricked into sterilized soil, in 12 inch × 18 inch, wire-bottomed flats (50 plants per flat). When the plants are ready for inoculation they are placed in a moisture chamber maintained at a temperature of 55° to 60° F., stated to be the optimum temperature for infection by *P. infestans*. The moisture chamber is of glass construction resembling a miniature greenhouse with hinged sides and will accommodate 20 flats. The flats are randomized in the chamber on a false floor of slat construction. A two-inch layer of moist sphagnum moss beneath the floor serves to keep the humidity at a relatively high level. Additional humidity is provided when needed by an atomizer spray. The inoculum consists of a spore suspension of *P. infestans* in distilled water made up at the swarm spore stage. The inoculum is sprayed over the plants with a hand syringe at the rate of approximately 50 cc. per flat. Primary infection takes place within 48 hours, but experience has shown that, for the disease to progress satisfactorily, it is necessary to leave the plants in the moist atmosphere of the chamber for at least five days after inoculation. An approximate time schedule for the various steps is as follows:

1. Tomato seed sown in shallow pots.
2. 12 to 15 days after sowing—the plants are pricked into flats.
3. 35 to 40 days after sowing (plants 4 to 5 inches tall and in 4- to 6-leaf stage)—inoculation is made.
4. 38 to 43 days after sowing—initial disease symptoms begin to appear.
5. 40 to 50 days after sowing—plants are examined and classified according to their reaction to the disease.

When the plants are examined for disease infection each plant is classified within the range 0 to 5 according to its reaction to the disease. These reaction classes are:

0. Complete freedom from disease symptoms.
1. Trace of infection—one or two to a few small necrotic spots.
2. Slight infection—many spots, but each separate and small.
3. Moderate infection—many spots, each separate and large.
4. Severe infection—many spots coalesced.
5. Complete killing.

Where a strain of tomatoes is reasonably homozygous in its reaction these individual plant ratings are totalled to give a whole sample rating on the basis of 100 plants; thus a sample rating may be from 0 to 500.

RESULTS OF PRELIMINARY EXPERIMENTS, 1948-50

Test 1, 1948-49

The first large-scale test was carried out during November and December 1948, and consisted of 10 strains of tomatoes which are shown, along with their reaction, in Table 1. The F_2 populations showed no apparent segregation for degree of susceptibility.

Test 2, 1948-49

This test included *L. peruvianum* subsp. *dentatum* and nine varieties of *L. esculentum*. Included in addition were two or three plants of each of an F_1 hybrid Sparx × *L. hirsutum*, *L. hirsutum* and *Solanum demissum*.

TABLE 1.—REACTIONS OF 8 SELECTIONS AND 2 VARIETIES OF TOMATOES TO INOCULATION WITH *P. infestans*

Record No.	Variety, selection, or parentage	Reaction
TB 48-1	Master Marglobe	351
*TB 48-2	Stokesdale	335
TB 48-3	*L. pimpinellifolium* × Sparx F_2	360
TB 48-4	*L. pimpinellifolium* × Pritchard F_2	364
TB 48-5	*L. pimpinellifolium*	382
TB 48-6	*L. pimpinellifolium* × San Rais F_2	336
TB 48-7	*L. pimpinellifolium* × Sparx F_2	374
TB 48-8	Ottawa TO-4 × *L. pimpinellifolium* F_2	352
TB 48-9	San Rais × Ottawa TO-3 F_2	313
TB 48-10	San Rais × *L. pimpinellifolium* F_2	330

* Susceptible check variety.

Examination showed appreciable differences in reaction to the disease. The majority of the samples were very susceptible, although the variety Devon Surprise and the South American type *L. peruvianum* subsp. *dentatum* showed less injury than any of the other normal samples tested. The reactions of the normal samples are shown in Table 2.

TABLE 2.—REACTIONS OF 9 VARIETIES AND ONE SUB-SPECIES OF TOMATOES TO INOCULATION WITH *P infestans*

Record No.	Variety, selection, or parentage	Reaction
*TB 49-1	Stokesdale	386
TB 49-2	Diener's Ventura	377
TB 49-3	Jan-ru	337
TB 49-4	*L. peruvianum* subsp. *dentatum*	291
TB 49-5	Danmark	306
TB 49-6	Devon Surprise	298
TB 49-7	Danmark Export	349
TB 49-8	Westlandia	349
TB 49-9	Vahle Leader	360
TB 49-10	Longred	323

* Susceptible check variety.

The ratings on the small samples are of interest. These were more mature plants than those in the normal samples and therefore cannot be compared directly with them. Their approximate reactions were as follows:

048-G-181	Sparx × *L. hirsutum*	200
048-380	*L. hirsutum*	100
049-G-101	*Solanum demissum*	Slight trace

The apparent resistance of *L. hirsutum* is in all probability a mechanical resistance due to the extremely pubescent character of the plants and fruit which would tend to hinder infection by the blight spores. This same dense hairiness is found in the hybrid Sparx × *L. hirsutum.* The wild potato species *S. demissum* was practically immune, showing only very small lesions which were very few in number.

Test 3, 1948-49

This test of 10 standard varieties of tomatoes included the varieties Southland and Lakeland, both reputed to have some resistance to late blight. Infection varied among the test samples from moderate to severe. Southland and Carter's Sunrise showed slower developing and less severe symptoms than the other varieties but could not be classed as resistant. Lakeland was definitely susceptible. Table 3 shows the results of this test.

TABLE 3.—REACTION OF 10 VARIETIES OF TOMATOES TO INOCULATION WITH *P. infestans*

Record No.	Variety, selection, or parentage	Reaction
*TB 49-11	Stokesdale	396
TB 49-12	Tatinter	373
TB 49-13	Mortgage Lifter	366
TB 49-14	Wood's Red	346
TB 49-15	Selandia	326
TB 49-16	Carter's Sunrise	304
TB 49-17	Red Jacket	368
TB 49-18	Ohio W-R Globe	321
TB 49-19	Southland	300
TB 49-20	Lakeland	354

* Susceptible check variety.

Test 4, 1948-49

In this test the level of infection was slightly lower than in Test 3, probably because of the difficulty in maintaining the desired cool temperature in the moisture chamber during the month of April when the samples were inoculated. The test included three Ottawa selections which had shown some resistance to *Alternaria solani* (early blight) under natural infection in the field. The Devon Surprise × Abel selection showed only slight infection, which is of interest in view of the fact that Devon Surprise showed evidence of slight resistance in Test 2. Two varieties, Tuckstir and Tuckcraig, were in a similar class to the Devon Surprise × Abel selection. Results are shown in Table 4.

TABLE 4.—REACTIONS OF 3 SELECTIONS AND 7 VARIETIES OF TOMATOES TO INOCULATION WITH *P. infestans*

Record No.	Variety, selection, or parentage	Reaction
*TB 49-21	Stokesdale	319
TB 49-22	Danmark × Abel	269
TB 49-23	Ottawa TO-19 (Danmark × *L. pimpinellifolium*)	283
TB 49-24	Devon Surprise × Abel	220
TB 49-25	Tuckstir	215
TB 49-26	Chih-mu-toa-me	318
TB 49-27	Florida Purple Stem	302
TB 49-28	Allred	305
TB 49-29	Schtambuvy	302
TB 49-30	Tuckcraig	244

* Susceptible check variety.

FIGURE 1. Tomato plants from Test 5 photographed twelve days after inoculation. In the lower photograph Stokesdale TB 50-8 is at the *left* and shows almost complete killing. At the *right*, Philippine No. 2 S_1 TB 50-1 shows marked resistance. The upper photographs are close-up views of the same plants with Philippine TB 50-1 at the top and Stokesdale beneath.

Also included in this test was a single, potted plant known as Philippine No. 2, grown from seed received from the Philippines in 1948. Two plant and fruit types had been noted among the plants grown from this seed and had been designated No. 1 and No. 2. One plant of each type was inoculated with *P. infestans*. Philippine No. 1 proved susceptible, while Philippine No. 2 appeared to be immune. The inoculation was repeated on No. 2 with similar results. The plant was propagated vegetatively and was re-inoculated in this test. This time it showed very slight disease symptoms on the leaves only.

Both Philippine No. 1 and Philippine No. 2 were propagated by seed and by cuttings. The seedlings were grown in the field in 1949 but were not subjected to late blight infection. This material proved to be highly heterozygous for plant and fruit characters and seed was then retained from individual plants for further greenhouse testing. During the same season seeds of three more strains of tomatoes were obtained from the Philippines, and were designated Philippine No. 4, No. 5, and No. 6. These were grown in the field and seed was obtained from individual plants for inoculation tests. These various selections from the Philippine material were then used as the basis of the 1949-50 tests.

Test 5, 1949-50

In this and subsequent tests carried on in the fall and winter of 1949-50 two moisture chambers were used with a total capacity of 2,000 plants. In Test 5 a start was made on separating out any resistant material that might be present in the seedlings of the various Philippine strains. The number of plants of each strain tested varied to some extent with the amount of seed available. Because of the promising performance of the original Philippine No. 2 plant, 500 S_1 seedlings of this plant were tested. Results are shown in Table 5.

TABLE 5.—REACTIONS OF SEEDLINGS OF 6 SELECTIONS OF PHILIPPINE TOMATOES TO INOCULATION WITH *P. infestans*

Record No.	Variety or selection	No. of plants	Reaction
TB 50-1	Philippine No. 2 S_1	500	133
TB 50-2	Philippine No. 2 S_2	250	303
TB 50-4	Philippine No. 1 S_1	200	306
TB 50-5	Philippine No. 4 S_2	200	162
TB 50-6	Philippine No. 5 S_2	200	166
TB 50-7	Philippine No. 6 S_2	200	314
*TB 50-8	Stokesdale	450	452

* Susceptible check variety.

The S_1 seedlings from the original Philippine No. 2 plant showed fairly good resistance but the sample TB 50-2, consisting of the S_2 generation from Philippine No. 2, proved susceptible. This may possibly be due to the fact that this sample originated from a very small field grown S_1 population which was quite heterozygous for plant and fruit type and in which the parental Philippine No. 2 type was not recovered. The S_1 seedlings

from Philippine No. 1 and the S_2 seedlings of Philippine No. 6 were susceptible while the S_2 seedlings of Philippine No. 4 and No. 5 were quite resistant. In spite of the fact that they were produced from heterozygous material, there was no evidence of segregation for resistance to the disease within these selections of the Philippine types.

Test 6, 1949-50

In this test the progeny of single plant selections of Philippine No. 4, No. 5, and No. 6 were checked against Stokesdale, each sample lot consisting of 200 plants.

As shown in Table 6, selections of No. 4 and No. 5 showed heavier infection than did their sister lines in Test 5. Selections of No. 6 reacted similarly to the No. 6 selection in Test 5. This might indicate either slight genetic differences in susceptibility between the different selections or it might be due to environmental conditions. Growth conditions were rather poor for this group of plants. Many plants were underdeveloped and were still rather small at the time of inoculation. This may have had an effect on the reaction readings, causing a somewhat higher level than expected.

Test 7, 1949-50

This test included 100 plants each of 4 Philippine selections and 13 varieties. In addition there were two pots each of *Solanum nigrum* and *Cyphomandra betacea* (*Solanum fragrans*). Certain difficulties, however, made it impossible to adhere to the schedule outlined under "Materials and Methods". As a result there was some possibility of incomplete inoculation and infection, and, therefore, reaction ratings for this test were not comparable to those in other tests. The varieties and selections tested are roughly grouped according to degree of infection as follows:

Trace to slight infection	TB 50-40	Philippine No. 2 S_1
	TB 50-41	Philippine No. 2 S_2
	TB 50-42	Philippine No. 4 S_2
	TB 50-43	Philippine No. 4 S_2
	TB 50-47	Champion Original
	TB 50-59	*Solanum nigrum*
	TB 50-60	*Cyphomandra betacea*
Moderate infection	TB 50-45	Ailsa Craig
	TB 50-46	Berg's Special
	TB 50-48	Tuckqueen
	TB 50-51	Rutgers
	TB 50-56	Bison
	TB 50-57	Break o'Day
Severe infection to complete killing	TB 50-39	Stokesdale
	TB 50-52	Pritchard
	TB 50-53	Early Rutgers
	TB 50-54	Alaska
	TB 50-55	Geneva John Baer
	TB 50-58	Bonny Best Super-Standard

Although results were not too accurate this test did serve to show the relative susceptibility of the varieties and strains included in it. It is noteworthy that the selections from Philippine No. 2 and No. 4 were again

TABLE 6.—REACTIONS OF 9 SELECTIONS OF PHILIPPINE TOMATOES TO INOCULATION WITH *P. infestans*

Record No.	Variety or selection	Reaction
*TB 50-9	Stokesdale	438
TB 50-10	Philippine No. 4 S_2	283
TB 50-11	Philippine No. 5 S_2	259
TB 50-12	Philippine No. 5 S_2	306
TB 50-13	Philippine No. 5 S_2	290
TB 50-14	Philippine No. 5 S_2	285
TB 50-15	Philippine No. 6 S_2	302
TB 50-16	Philippine No. 6 S_2	289
TB 50-17	Philippine No. 6 S_2	297
TB 50-18	Philippine No. 6 S_2	305

* Susceptible check variety.

outstanding. The two species *Solanum nigrum* and *Cyphomandra betacea* both developed slight leaf lesions which did not spread readily, indicating that they may possess some resistance to the disease.

The Philippine material used in these tests was obtained in 1948 from Simon B. Barbadillo, Bangued, Abra, Philippine Islands. The most promising is the one designated Philippine No. 2.

It belongs to the species *L. esculentum* and is one of a number of strains known in the Philippine Islands as Ilocano tomatoes. The characters are not entirely fixed and segregation is quite marked. In general, however, the plants are indeterminate, variable in height (14 to 26 inches) and spread (36 to 50 inches). Foliage is moderately dense with numerous long branches and medium long internodes. The leaves are medium in size (8 to 11 inches). The margins are dissected sharply but sparsely and not deep; the surface is dark green, smooth and somewhat glossy.

The fruit size is highly variable, 2.5 to 3.6 inches equatorial diameter and 1.3 to 1.6 inches polar, giving them a definite flat appearance, extremely fasciated and with a deep stem cavity. The shoulders are prominent, heavily ribbed, the stylar scar is also large, deep and ribbed. Usually the centre is corky, the locules are scattered towards the outer areas in the lobes between the ribs. The colour is medium red with greenish streaks. Usually there are four fruits per truss. Apart from some resistance to *Phytophthora infestans*, Philippine No. 2 has little horticultural value.

This series of tests has been of a preliminary nature only and no conclusions are set forth regarding the resistance or susceptibility of all the varieties and strains tested. Further testing is required to confirm the preliminary results and to select any resistant lines from what appear to be moderately heterozygous types.

ACKNOWLEDGMENT

The writers express their appreciation to L. T. Richardson, formerly of the Division of Botany and Plant Pathology, for his assistance and co-operation in the early stages of this work.

REFERENCES

1. Alexander, L. J., and V. Wright. A survey of the genus *Lycopersicon* for resistance to the important tomato diseases occurring in Ohio and Indiana. U.S.D.A. Plant Disease Reporter Suppl. 136. 1942.
2. Andrus, C. F. Resistance of tomato varieties to late blight in South Carolina. U.S.D.A. Plant Disease Reporter 30 : 269. 1946.
3. Andrus, C. F., and G. B. Reynard. Relative resistance of tomato varieties, selections and crosses to defoliation by *Alternaria* and *Stemphylium*. U.S.D.A. Circ. 652. 1942.
4. Berkeley, G. H., and J. K. Richardson. Tomato diseases. Canada Dept. Agr. Pub. 759. 1944.
5. Boswell, V. R., and E. Bostelmann. Our vegetable travelers. Nat. Geographic 46 : 145. 1949.
6. Doolittle, S. P. Tomato diseases. U.S.D.A. Farmers' Bull. 1934. 1943.
7. Epps, W. M. Tomato late blight in South Carolina. U.S.D.A. Plant Disease Reporter 30, Suppl. 165, p. 310. 1946.
8. Heald, F. D. Manual of plant diseases, 2nd Edition. McGraw Hill, New York. 1933.
9. Locke, S. B. Resistance in South American *Lycopersicon* species to early blight and Septoria blight. Phytopath. 32 : 12. 1942.
10. Luckwill, L. C. The evolution of the cultivated tomato. J. Roy. Hort. Soc. 68 : 19. 1943.
11. Luckwill, L. C. The genus Lycopersicon. Aberdeen University Studies No. 120. 1943.
12. Moore, W. D., and G. B. Reynard. Varietal resistance of tomato seedlings to the stem lesion phase of *Alternaria solani*. Phytopath. 35 : 933. 1945.
13. Muller, C. H. A revision of the genus *Lycopersicon*. U.S.D.A. Misc. Pub. 382. 1940.
14. Porte, W. S., and F. L. Wellman. Development of inter-specific tomato hybrids of horticultural value and highly resistant to Fusarium wilt. U.S.D.A. Circ. 584. 1941.
15. Porte, W. S., and H. B. Walker. A cross between *Lycopersicon esculentum* and disease resistant *L. peruvianum*. Phytopath. 35 : 931. 1945.
16. Porter, D. R., and J. H. MacGillivray. The production of tomatoes in California. Calif. Agr. Ext. Service Circ. 104 (revised). 1942.
17. Richards, M. C., and R. W. Barratt. A partial survey of the genus *Lycopersicon* for resistance to *Phytophthora infestans*. U.S.D.A. Plant Disease Reporter 30 : 16. 1946.
18. Strong, M. C. Tomato diseases in Michigan. Circ. Bull. 139 (2nd revision), Mich. Agr. Exp. Sta. 1943.
19. Young, P. A., and J. W. MacArthur. Horticultural characters of tomatoes. Texas Agr. Exp. Sta. Bull. 698. 1947.

STUDIES IN RED CLOVER SEED PRODUCTION[1]

R. M. MacVicar[2], E. Braun[3], D. R. Gibson[4], and C. A. Jamieson[5],

Experimental Farms Service, Canada Department of Agriculture, Ottawa

[Received for publication June 25, 1951]

Low acreage yields are the rule in red clover seed production. Occasionally, under very favourable conditions, substantial yields are obtained; but in most years yield per acre does not exceed 100 pounds. Seed yield is influenced by many factors. Some of these cannot be controlled, but the controllable ones may have a marked influence on seed yield. A joint project initiated at the Central Experimental Farm at Ottawa in 1950, by the Divisions of Apiculture and Forage Plants, is concerned with the control of harmful insects and the utilization of honeybee pollinators in red clover seed production. This paper purports to present the data obtained during the season of 1950.

CONTROL OF INJURIOUS INSECTS

The control of injurious insects in legume seed production has developed to such an extent, in recent years, that it is now adopted as general practice in many of the most important seed areas. While the amount of detailed experimental work has been limited, field trials have been extensive enough to establish beyond question the value of such control in crops such as alfalfa, white clover and alsike. This has not been the case with red clover. Wilsie (15) did not obtain significant results from insect control in red clover, while Arnott (2) reported that the results he obtained did not justify insecticide control recommendations. Medler *et al.* (10), on the other hand, reported appreciably higher yields of red clover seed when insects were controlled. The results obtained from field experiments in the Ottawa area in 1950 are reported here.

Experimental Procedure

An area of about 68 acres of second growth Ottawa red clover was utilized for experimental trials. The first growth was removed by a forage harvester for dehydration purposes, during a 6-day period beginning June 15th. Prior to this cutting, insect sweeps were made to determine which injurious insects were present. Four treatments and a control were used and applications were made on a basis of 5 replications. The area assigned to each replicate was not uniform due to the field layout, but in no case was a replicate imposed on an area less than 8 acres in size.

Treatment 1. DDT applied just prior to bloom.
Treatment 2. DDT applied just prior to bloom and toxaphene at full bloom.
Treatment 3. DDT applied just prior to bloom and methoxychlor at full bloom.
Treatment 4. DDT plus toxaphene applied just prior to bloom.
Treatment 5. Control.

[1] Joint contribution from the Divisions of Forage Crops and Apiculture, Experimental Farms Service, Canada Dept. of Agriculture, Ottawa.
[2] Senior Agronomist—Division of Forage Plants, Central Experimental Farm, Ottawa.
[3] Senior Apiculturist—Division of Apiculture, Central Experimental Farm, Ottawa.
[4] Technical Officer—Division of Forage Plants, Central Experimental Farm, Ottawa.
[5] Dominion Apiculturist—Division of Apiculture, Central Experimental Farm, Ottawa.

Applications were made as sprays at a pressure of 225 lb. p.s.i. in approximately 50 gallons of water per acre. Rate of chemical application was as follows:

DDT—(25% emulsion)—1.5 lb. active ingredient per acre.
Toxaphene—(50% emulsion)—1.5 lb. active ingredient per acre.
Methoxychlor—(50% wettable powder)—1.5 lb. active ingredient per acre.

All applications were made without regard to the time of day. This may not have been desirable but inclement weather conditions made it unavoidable. Data determinations were made by extensive sampling. Twenty samples from one square yard areas were drawn from each of the treatments in all replicates.

Results

By making use of the random samples drawn from each treatment for each replicate it was possible to establish levels of significance within each replicate and for the whole experiment. Table 1 gives the summarized data and L.S.D. values for all treatments and replicates.

An over-all average figure of 134 lb. of seed per acre was obtained from the control plots. Significant mean square values were obtained for treatments 1, 2, and 4 when compared with the control, while treatment 2 showed a significant value over treatment 3.

When the replicates are considered as individual fields, a somewhat contradictory picture is obtained. In replicate 1, treatments 1 and 2 were significantly higher than the others. Treatment 2 showed an increase of approximately 47 per cent over the check. In replicate 2, treatment 2 was the lowest, due to some condition not readily apparent. The reduced yield in this area may be accounted for by the treatment falling on a low-lying portion of the field where considerable winter injury had occurred. This assumption is based on the variable yields, ranging from 27 lb. to 202 lb. per acre, obtained from samples taken over this treatment. In

TABLE 1.—YIELDS OF RED CLOVER UNDER DIFFERENT INSECTICIDE TREATMENTS (LB. PER ACRE)

Spray Treatment	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	All Replicates
T1. DDT—pre-bloom	211	109	156	123	107	153
T2. DDT—pre-bloom; Toxaphene—full-bloom	229	100	211	145	103	170
T3. DDT—pre-bloom; Methoxychlor—full-bloom	132	206	200	142	73	147
T4. DDT—pre-bloom; Toxaphene—pre-bloom	170	116	250	106	121	155
T5. Control	156	153	152	96	95	134
General Mean	179	137	194	123	99	152
L.S.D. (P = .05)	36	43	57	37	None	18

replicate 3, treatments 2, 3 and 4, are significantly higher than treatment 1 and the check. In replicate 4, treatments 2 and 3 are higher than the check while in replicate 5 there is not significant difference between treatments.

By a rearrangement of the seed yield data it was possible to obtain a measure of the effectiveness of toxaphene in controlling a rather heavy grasshopper infestation. On analysis of the data it was found that areas not treated with toxaphene gave yields of about 126 lb. per acre, a figure that was significantly lower than the 150 lb. per acre obtained from the toxaphene-treated plots.

A survey made of the insect population in the first growth of red clover on the experimental area revealed the presence of a rather diverse population of insects. The clover head caterpillar, *Grapholitha interstinctana* (Clem.) the clover head weevil, *Tychius stephensi* (Schonh.), the head weevil, *Hypera meles* (F) and the clover leafhopper *Aceratagallia sanguinolenta* (Prov.), considered of importance in legume seed production, were present in varying numbers.

A survey made just previous to spraying revealed the presence of substantial numbers of clover head weevil, *Tychius stephensi* (Schonh), clover root curculio, *Sitona hispidula* (F) and grasshoppers *Melanoplus Spp.* Later surveys made after the spray applications indicated marked decreases in the populations of these insects.

Discussion

It is apparent from the data presented here that the use of insecticides has resulted in substantial yield increases. When the difference between treatment 2 (DDT at the pre-bloom stage and toxaphene at full bloom) and the control is expressed in per cent, a yield difference of approximately 25 per cent is found. Treatment 4, involving a single combined application of DDT and toxaphene, gave a yield within 15 lb. of treatment 2. Since this treatment involves only a single application it is worthy of further consideration. There is a possibility that spraying could be delayed until the half-bloom stage so that this treatment could give better over-all control. The possible deleterious affect on pollinators of applying a spray at such a time would have to be assessed before it could be recommended.

The fact that the toxaphene-sprayed area gave approximately 18 per cent greater yield than areas not so treated is a matter of considerable interest. It would appear that grasshoppers alone can bring about marked decreases in seed yield in red clover.

It will be noted that spray applications in this experiment were at high volume and pressure. Low volume spraying and dust applications have not been made; so their comparative effectiveness cannot be discussed at this time.

POLLINATION

The role of insect pollinators in legume seed production is now well established. For some time there were widely divergent views as to the effectiveness of honeybees as pollinators in red clover but it is now generally accepted that honeybees are efficient pollinators of this crop. Stapel (13), Goetze (7), Butler (5), Hopkins (8), Richmond (12), Dunham

(6) and others have demonstrated the effectiveness of domestic bees in working red clover. Detailed observations made by the United States Department of Agriculture over a 3-year period indicated that 82 per cent of pollination in red clover was performed by honeybees.

The foraging force of the honeybee colony, from the pollination standpoint, is divided into pollen- and nectar-gatherers. Parker (11) has shown that 25 per cent of the Italian bees specialize in pollen collecting, 58 per cent in nectar collecting and 17 per cent collect both nectar and pollen on the same trip. A higher proportion of pollinating insects in red clover will be governed by (1) the tongue length of the pollinator insect, (2) the length of the corolla tube, (3) the height of the nectar column in the corolla tube and (4) the actual reaching distance of the insect into the head of the individual floret. Honeybee tongue lengths range from 5.8 to 6.4 mm., while that of the bumblebee ranges from 5 to 14 mm., depending upon the species being studied. Corolla tube length varies from 8 to 9.6 mm., depending on the variety of red clover. The height of the nectar column may on occasion reach 5 mm. or more. Martin (9) found that the honeybee can push its head approximately 1 mm. into the red clover floret and thus extend its reaching distance by that amount.

This study was concerned with:—

1. The pollen-gathering activities of the honeybee on red clover in the presence of competing crops.
2. The relationship of flight distances of honeybees to seed yield.
3. The population density of honeybees as compared to that of native pollinators such as bumblebees.
4. The feasibility of stimulating pollinator activity by artificial means.

Experimental Procedure

A. Honeybee colonies within a radius of $1\frac{7}{8}$ miles from the experimental area were eliminated to provide some measure of control of the honeybee population in the area.

B. Sixty-eight colonies of honeybees were moved into the area on July 28, 1950, immediately after spraying with insecticides and placed at the southerly end of a 68-acre rectangular field of second growth "Ottawa" red clover. At this time approximately 15 per cent of the clover was in bloom. Five areas, located at distances of 200, 500, 833, 1126 and 1426 yards respectively from the apiary, were marked out as stations on which observations could be made. The colonies remained in the field until September 21.

C. Ten colonies were provided with pollen traps and collections were made daily. The pollen was oven-dried, weighed and a sample amounting to 2 per cent of the total weight of pollen gathered by each colony was taken for analysis. A technique to determine the number and kinds of pollen grains present was devised as follows:—

The pollen pellets were dispersed gently with a mortar and pestle, washed into a 100 ml. volumetric flask with 25 ml. of water and diluted to the mark with a solution of 2.5 per cent sodium alginate. The increased viscosity thus attained held the pollen grains suspended long enough to permit composite sampling. The number and kinds of pollen grains present were determined in 20 squares of a haemocytometer counting cell. Six

such counts were made from each sample to determine the daily mean. Due to the time-consuming nature of these determinations, counts were made on only 3 representative colonies.

D. Dead bee traps were installed on 5 colonies and collections made daily to ascertain the possible lethal effect on honeybees of the various insecticide sprays applied.

E. One-minute pollinator counts covering honeybees and bumblebees were made twice daily at 9 a.m. and 1.30 p.m. on a 2-square yard area in randomized plots in 25 designated locations. Observations indicated few other types of pollinators present in the area.

F. Dilute honey sprays (1½ pounds of liquefied honey to 3 gallons of water) were applied to small areas (50 square feet) on each station, and insect population counts made to ascertain the effect of the attractant on the pollinators.

G. Relevant seed yield data were obtained by the method outlined in the first part of this paper.

Results

1. Placement of Colonies.

In order that the relationship between flight distance and honeybee activity might be studied the entire apiary of 68 colonies was placed in one location at the south end of the experimental area. Subsequently the area was arbitrarily divided into 5 stations whose distances from the apiary ranged from 200 to 1426 yards. By using the sampling procedure mentioned previously, yield data as outlined in Table 2 were obtained.

TABLE 2.—THE EFFECT OF COLONY PLACEMENT ON RED CLOVER SEED YIELD AND POLLINATOR ACTIVITY

Distance from apiary (in yards)	Yield of clean seed per acre in lb. (Means of all observations)	Pollinators per 2 sq. yd. area (Means of 60 observations)
200	139	7.47
500	201	8.82
833	132	6.83
1126	142	7.75
1426	100	4.27
L.S.D. (P = .05)	20	No. Sign.

It will be observed that the yield at 500 yards from the apiary was significantly higher than that for all other distances. It is probable that the lower yield obtained at 200 yards was due to the relatively poor stand of clover that existed at that station. This assumption was supported by the general seed yield obtained in this area. It will be noted that similar results were obtained at 833 and 1126 yards while the yield at 1426 yards was significantly lower than at all other distances. Pollinator counts appear to be in direct relationship to the seed yield obtained.

2. Pollinator Activity.

The flight activity of pollinators is directly influenced by numerous factors such as temperatures, rain, sunlight, wind velocity, etc. Data obtained in this study are presented in Table 3.

TABLE 3.—VARIATION IN POLLINATOR ACTIVITY AS TO DAYS AND TIME OF DAY, AND RATIO OF HONEYBEES TO BUMBLEBEES

Items	Means of 150 observations (Counts on basis of 2-square yard area)				
	Range of pollinator activity	All Pollinators		Bumble-bees	Honey-bees
		A.M.	P.M.		
Aug. 1 to Aug. 21	15.35 to 2.35				
Time of day		5.27	8.77		
Type of pollinator				1.47	12.58

As might be expected, pollinator activity varied widely from day to day. There was considerably greater activity in the afternoon than in the morning. The honeybee population, on the average, was about eight times greater than that of bumblebees.

3. Pollinator Training.

As outlined under procedure *F*, an effort was made to attract honeybees to clover locations by means of a honey spray. The effectiveness of such a spray as an attractant was indicated by the counts made 3 hours and 5 hours after application. The bee population at the 3-hour interval was so dense on the sprayed plots that accurate counts could not be made. The activity at the 5-hour interval had decreased considerably although it was still much greater than in the unsprayed area. Counts made on these plots two days later indicated a normal population. It was observed that the bees gathered the honey as readily from the leaf and stem surfaces as from the blooms. Bumblebees were evidently not attracted in exceptional numbers to the sprayed plots.

TABLE 4.—SEED YIELD AS INFLUENCED BY A HONEY SOLUTION APPLIED AS AN ATTRACTANT FOR POLLINATORS

Treatment	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Mean
Honey Spray	139	201	132	142	100	143
Control	136	197	144	149	95	144

The seed yields on the honey-sprayed plots were not influenced by the increased pollinator activity which was only of a transitory nature.

4. Pollinator Mortality.

The honeybees under observation ranged over the same area used for the insect control work described in the first part of this paper. It was found that the daily mean death rate of honeybees from 5 colonies, over the 15-day period, was 14.6, 28.3, 12.1, 18.9, and 22.9 respectively.

Table 5.—Analysis of pollen taken in pollen traps, indicating sources in per cent (mean of six counts)

Date August	Colony No. 69				Colony No. 155				Colony No. 126			
	Red Clover	Buck-wheat	Sow Thistle	Others	Red Clover	Buck-wheat	Sow Thistle	Others	Red Clover	Buck-wheat	Sow Thistle	Others
3	6.6	85.5	2.4	5.5	2.2	72.2	7.7	17.9	5.0	67.5	6.4	21.1
5	42.1	31.8	12.7	13.4	12.0	28.8	30.7	29.5	21.6	31.4	15.6	31.4
7	44.5	12.8	32.3	10.4	31.6	16.0	37.1	15.3	19.1	18.2	35.9	26.8
9	55.2	31.6	6.2	7.0	23.7	59.5	9.6	7.2	18.8	60.9	6.2	14.1
11	48.1	25.2	10.7	16.0	36.6	23.0	26.5	13.9	17.6	29.7	21.6	31.1
13	62.8	11.6	8.5	17.1	68.3	15.9	9.0	6.8	47.3	30.6	3.8	18.3
15	36.8	47.6	6.5	9.1	55.1	36.5	5.3	3.1	47.7	35.6	0.0	16.7
17	63.1	18.3	4.2	14.4	72.0	23.2	0.6	4.2	42.6	34.5	2.5	20.5
Mean	44.9	33.1	10.4	12.8	37.7	35.0	15.8	12.1	27.7	38.5	11.5	22.5

There were daily fluctuations due to flight conditions as influenced by existing weather conditions, but no abnormal losses that could be attributed to the spray materials used was recorded. Examination of the colonies revealed no apparent loss of brood due to poisoning of accumulated pollen stores.

5. Pollen Collections.

In Table 5 data obtained from the analysis of pollen material gathered from three representative colonies are tabulated. The major portion of the pollen collected by these colonies was red clover and buckwheat in about equal amounts. There were rather extreme variations in collections from day to day and between the collections made by the different colonies on the same day.

Discussion

It would appear, with regard to placement of colonies, that pollinator effectiveness decreases at a point somewhere between 3/5 and 4/5 of a mile from the apiary location. Dispersal of colonies throughout a clover area would seem desirable if distances greater than those indicated are involved.

The wide variation known to exist in pollinator activity from day to day is borne out by the data presented here. Under the conditions of this experiment the bumblebees, because of their limited numbers, did not appear to make a very large contribution. It may be significant, however, that bumblebees were found working at times when honeybee activity was at a minimum. Their periods of greatest activity do not necessarily coincide with those of the honeybee.

From the limited amount of work done on pollinator training definite conclusions cannot be drawn. However, the work undertaken here did indicate that the use of honey sprays for this purpose was not likely to be of value.

Some importance may be attached to the observation that no abnormal mortality occurred when honeybees foraged over a rather extensive area that had been sprayed with insecticides. Since time of spraying for insect control is often influenced by the fear that harm will be done to the pollinating insects it is important that the lethal effects, if any, of the insecticides used be definitely established.

The fact that honeybees appear to make substantial collections of red clover pollen, even when other good pollen sources are available, commands attention. The opinion is widely held that the honeybee is partial to crops other than red clover. Dunham (6), Butler (3) and others have shown that the honeybee is an efficient and remarkably constant pollinator of red clover. The results obtained in this study corroborate this finding.

NECTAR SECRETION

Numerous workers in various countries have demonstrated that the honeybee can collect nectar from red clover especially when the nectar rises to 1.7 mm. or more above the ovaries of the flower. Research work in Denmark, Great Britain and the United States has revealed that, under hot and dry weather conditions, nectar in the red clover corolla tubes may

reach a height of 5 mm. over the ovary and thus be readily accessible to the honeybee. Butler (3) has found that 98 per cent of bumblebees working on red clover were collecting nectar, and that honeybees periodically store a surplus of nectar from this source. He examined the loads of 484 honeybees working red clover in detail and found that 60 per cent were nectar-gatherers and 40 per cent pollen-gatherers. This worker states that "so far as can be determined no accurate measurements of the volume of nectar contained in a flower in relation to honeybee activity have as yet been made".

The principal object of this study was to determine the relationship between volume and concentration of nectar in red clover as related to pollinator activity and seed yield.

Experimental Procedure

In making this study an attempt was made to process clover heads of the same maturity. Screen cages were used to ensure blooms not worked by bees. Each day heads from both outside and inside the cages were tagged when the first ring of florets were beginning to open. Five days after tagging the heads were ready for sampling. Samples were drawn from each of the sampling locations twice daily. At each sampling 3 heads were drawn from inside the cages and 3 heads from the outside area. Thus 30 heads were processed both morning and afternoon. The blooms with attached stems, as collected, were placed in vials containing distilled water. Readings from these heads were made as soon as possible after picking.

A simple technique was used for the extraction of nectar. Rings of florets from the central section of the blooms were chosen for sampling. The nectar was removed from the florets by squeezing the corolla tubes.

TABLE 6.—NECTAR READINGS FOR A 15-DAY PERIOD IN RED CLOVER (MEANS OF 20 OBSERVATIONS)

Date	Volume in microlitres per floret	Per cent solids
August 4	.360	37.0
August 5	.307	31.5
August 6	.329	54.3
August 7	.268	50.3
August 8	.287	50.6
August 9	.349	46.6
August 10	.328	51.7
August 11	.250	56.7
August 12	.267	60.6
August 13	.374	57.7
August 14	.393	56.5
August 15	.424	57.9
August 16	.458	52.9
August 17	.577	41.5
August 18	.542	43.7
General Mean	.368	50.0
L.S.D. at 5% point	.115	.31
C. V.	49.38 %	9.88 %

The droplet, as it gathered at the base of the tube, was fed to a small micropipette mounted on a stand. The number of florets required to provide a sufficient amount of nectar in the pipette for measurement purposes was recorded. The length of the nectar column in the tube, which was of known volume, was also recorded. It was possible to deposit the collected nectar on the prism of a Zeiss refractometer by applying pressure to one end of the pipette. By means of this technique data showing nectar volume and concentration were obtained.

Results

1. Daily and other variations in nectar secretion.

Daily readings were taken for volume and per cent solids for a period extending from August 4 to August 18.

Analysis of the data revealed that volume and per cent solids varied significantly between days. Data were obtained to check the effect of time of day, artificial conditions induced by enclosing red clover blooms by screen cages, and the use of insecticides on secretion of nectar. This is recorded in Table 7 which shows that the volume of nectar was significantly higher in the morning and concentration of nectar was higher in the afternoon. Blooms growing inside cages gave significantly higher values for both volume and concentration than did blooms growing without cover. The lower volume here might be partially explained by the possibility that bees may have removed nectar before sampling was done. This consideration would not affect concentration however. It is likely that a difference in humidity, reduced wind velocity and temperature within the

TABLE 7.—NECTAR READINGS AS RELATED TO TIME OF DAY, GROWTH LOCATION AND INSECTICIDES. (MEANS OF 150 OBSERVATIONS FOR TIME OF DAY AND LOCATION, 60 OBSERVATIONS FOR INSECTICIDES)

Item	Volume of Nectar			Per cent Solids in Nectar		
	Means per floret µls.	L.S.D. (p = .05) µls.	C.V. %	Means per floret %	L.S.D. (p = .05) %	C.V. %
Times						
9.30 a.m.	.391	.040	49.38	48.1	1.14	9.88
1.00 p.m	.345			51.8		
Locations						
Inside of cage	.485	.040	49.38	55.5	1.14	9.88
Outside of cage	.250			44.4		
Insecticides						
1. DDT prebloom	.369			51.5		
2 DDT prebloom Toxaphene full-bloom	.395			49.8		
3. DDT pre-bloom Methoxyclor fullbloom	.348			51.0		
4. DDT pre-bloom Toxaphene pre-bloom	.383			51.3		
5. Check	.343	None		46.1	1.8	9.88

TABLE 8.—HOURLY VARIATIONS OF PER CENT SOLIDS IN NECTAR OF RED CLOVER BLOSSOM

Hours	9th day	15th day	16th day	17th day	Mean
10 a.m.	52.5	60.1	62.1	45.0	54.9
11 a.m.	59.8	64.8	61.6	43.3	57.4
12 noon	53.0	68.3	62.7	46.3	57.6
1 p.m.	50.4	68.4	62.3	52.0	58.3
2 p.m.	54.8	68.4	56.3	48.6	57.0
3 p.m.	61.5	62.4	52.4	53.6	57.5
4 p.m.	58.3	67.9	62.4	61.6	62.6
Mean	55.75	65.75	59.97	50.05	57.9

cages, brought about a more favourable condition for nectar secretion. It should be noted that neither field location nor insecticides influenced nectar volume or concentration, although nectar concentration was significantly lower on Station 5 where the check was located.

Data on hourly variations were obtained on four days. The flowers processed were taken from a screen cage and were as uniform in maturity as choosing by observation would permit. On analysis it was found that volume variations were so extreme, and so lacking in pattern, that the data were worthless. These variations probably could be accounted for in large part by individual plant differences.

A trend to higher percentages of solids was found from morning to afternoon. This is shown by the data presented in Table 8. It is significant, however, that per cent solids vary considerably from day to day as indicated by the mean values presented.

2. Nectar secretion as affected by ovule fertilization.

Five pairs of unrelated heads, at the full-bloom stage and growing in screened cages were marked by tagging. By removing a row of florets over the crown of the blossom, the heads were delineated into two sections. Pollen from one section was used to cross-pollinate a section of the other member of the pair and vice versa. Thus reciprocal crosses were made. This procedure was followed with all five pairs. Pair No. 1 was used for data determinations on the third day following pollination, pair No. 2 on the fourth, and so on until on the seventh day after pollination the last pair was used.

Several points of interest are manifest in the data presented in Table 9:

1. Nectar flow in fertilized flowers diminished rapidly by the fifth day and the secretion was not measurable after that time.

2. Nectar flow in unfertilized flowers was still measurable seven days after full-bloom although beginning to diminish rapidly at that time.

3. Per cent solids in the nectar seemed to decrease as early as three days after pollination.

4. A marked decrease in per cent solids in nectar from unpollinated flowers was not apparent until about seven days after full-bloom.

5. Slight wilting was noticed in the florets as early as the third day after pollination while it was not apparent in unpollinated florets until six days after full-bloom.

TABLE 9.—A COMPARISON OF NECTAR VOLUME AND CONCENTRATION IN FERTILIZED AND UNFERTILIZED RED CLOVER FLOWERS

			Fertilized flowers			Unfertilized flowers			Average of reciprocals by fertilized and unfertilized pairs			
									Length of column		Total solids	
Date	Cross	Days after fertilization	Wilting	Length of column mm.	Total solids %	Wilting	Length of column mm.	Total solids %	Fert. mm.	Not fert. mm.	Fert. %	Not fert %
14 Aug./50	5 × 6	3	Slight	23.4	54.0	None	36.8	59.4	24.1	37.6	53.6	59.7
14 Aug./50	6 × 5	3	Slight	24.8	53.2	None	38.4	60.0				
15 Aug./50	3 × 4	4	Yes	21.5	60.5	None	25.5	65.5	23.7	31.5	58.3	64.7
15 Aug./50	4 × 3	4	Yes	26.0	56.1	None	37.5	60.0				
16 Aug./50	7 × 8	5	Yes	4.0	57.0	None	23.5	64.0	4.7	23.5	55.5	63.0
16 Aug./50	8 × 7	5	Yes	5.5	54.0	None	23.5	62.0				
17 Aug./50	9 × 10	6	Yes brown	0.0	—	Slight	19.5	45.0	0	18.5		55.5
17 Aug./50	10 × 9	6	Yes brown	0.0	—	Slight	17.5	66.0				
18 Aug./50	11 × 12	7	Yes brown	0.0	—	Yes brown	10.0	45.0		—		46.9
18 Aug./50	12 × 11	7	Yes brown	0.0	—	Yes brown	6.0	48.8	0			

Discussion

The nectar secretion is extremely variable and apparently the flow was conditioned by a great many factors. The possibility exists that the trend toward higher per cent solids in the afternoon may explain the greater pollinator activity at that time. However, the reduced volume of nectar in the afternoon, in theory, should reduce pollinator activity. It is noteworthy that on numerous occasions the honeybees were found to be visiting the very young clover florets. It is possible that, since the corolla tubes of these florets had not grown to full length, the nectar present was more readily available to the honeybees.

SUMMARY

1. Data from a co-operative project between the Division of Forage Plants and the Division of Apiculture, Central Experimental Farm, Ottawa, on red clover seed production with particular reference to harmful insect control, pollination and nectar secretion are given here.

2. Seed yields of red clover were increased by as much as 25 per cent when meadows were sprayed with insecticides to control harmful insects.

3. The most effective control was a spray of DDT at the pre-bloom stage and a second spray of toxaphene at the full-bloom stage.

4. When grasshoppers were controlled with toxaphene, seed yields were increased approximately 18 per cent.

5. Seed yield data revealed that pollinator effectiveness decreased at a point between 3/5 and 4/5 of a mile from the apiary.

6. Seed yield varied directly with pollinator activity.

7. Pollen grain counts from trapped samples indicated a dispersal of some pollen gatherers from the crop to be pollinated to competing crops. However, it is noteworthy that pollen-gathering activity in red clover was maintained at a high level.

8. The insecticides used did not appear to cause abnormal pollinator mortality.

9. Honeybees and bumblebees were present in a ratio of 12.5 to 1.5, when one colony of honeybees per acre was used.

10. Seed yields were not increased when honey was applied to the foliage as an attractant to pollinators.

11. The volume and concentration of nectar varied significantly between days, while during the day the volume was significantly higher in the morning and concentration was higher in the afternoon. Blooms in screen cages gave significantly higher values for both volume and concentration than uncovered blooms. Insecticide-sprayed plots showed significantly higher nectar concentration than the unsprayed (check) plots, but there was no significant difference in the volume of nectar between these plots.

12. The nectar volume in fertilized and unfertilized flowers diminished very rapidly after the fifth and seventh day, respectively. The third day after fertilization the per cent of total solids decreased, but this was not apparent in unfertilized flowers until seven days after full-bloom. Wilting followed a similar trend.

ACKNOWLEDGMENTS

The co-operation of W. Stevens, of the Entomological Division, Science Service, in making insect surveys was much appreciated. The careful and detailed work of technicians G. W. Mills, James Sherman, Johston Jack and Ernest Buckingham is also gratefully acknowledged.

REFERENCES

1. Armstrong, J. M., and C. A. Jamieson. Cross pollination of red clover by honeybees. Sci. Agr. 20 : 574-85. 1940.
2. Arnott, D. A. Rept. of Ont. Legume Research Committee. 1950.
3. Butler, C. G. A study of the frequency with which honeybees visit red clover (Trifolium pratense) together with an examination of the environmental conditions. Ann. of Appl. Biol. 28 : 125-134. 1941.
4. Butler, C. G., E. P. Jeffree, and H. Kalmus. The behaviour of honeybees on an artificial and on a natural crop. J. Exp. Biol. 20 : 65-73. 1943.
5. Butler, C. G. The influence of various physical and biological factors of the environment on honeybee activity. An examination of the relationship between activity and nectar concentration and abundance. J. of Exp. Biol. 21 : 5-12. 1945.
6. Dunham, W. E. Collecting red clover pollen by honeybees. J. Ec. Ent. 32 : 668-70. 1939.
7. Goetze, G. Versuche zur Ausnutzung des Rotkleis durch die Honigbeine. Beetr. Agrawissensch. 2 : 35-49. 1948.
8. Hopkins, I. History of the bumblebee in New Zealand: Its introduction and results. Bull. 46, New Series, N.Z. Dept. of Agr., Industries and Commerce. 1914.
9. Martin, J. N. Why the high price of red clover seed? Report of the State Apiarist for Iowa. 1937.
10. Medler, J. T., and T. K. Chamberlin. Seed yields of red and Ladino clovers increased by use of insecticides. J. Econ. Ent. 41 : 108. 1948.
11. Parker, R. L. The collection and utilization of pollen by the honeybee. Cornell Univ. Agr. Exp. Sta. Mem. 98. 1926.
12. Richmond, R. G. Red clover pollination by honeybees in Colorado. Colo. Agr. Expt. Sta. Bull. 391, pp. 1-22. 1932.
13. Stapel, C., and K. M. Erickson. Investigation by means of pollen analysis on the relation between honeybees (A. mellifica L. and A. mellifica var. legustica spinola) and red clover. Tiddsskr Planteavl. 41 : 487-592. 1936.
14. U.S.D.A. Annual report for 1949 (Bee Culture Section).
15. Wilsie, C. P. Producing alfalfa and red clover seed in Iowa. J. Amer. Soc. Agron. 4 : 345. 1949.

A DETERMINATIVE STUDY OF AMYLOLYTIC, STENOTHERMOPHILIC BACTERIA ISOLATED FROM SOIL[1]

EGON STARK[2] AND P. A. TETRAULT[3]

Laboratories of Bacteriology, Department of Biological Sciences, Purdue University, Lafayette, Indiana

[Received for publication June 19, 1951]

INTRODUCTION

The streamlined version of the genus *Bacillus* by Smith, Gordon and Clark (12) was incorporated into Bergey's Manual of Determinative Bacteriology (2). It was put to a severe test by Knight and Proom (8) and was found to be very satisfactory. However, the section on thermophilic species remained poorly defined. In 19 9, Gordon and Smith (6) described stenothermophilic bacteria, most of which belonged to one species, *Bacillus stearothermophilus* Donk. Consequently, they proposed a "species' pattern", emending the original description given by Donk (4) in 1920.

During an investigation, dealing with the breakdown of starch by bacteria at high temperatures (14), 34 cultures were isolated from muck soil of Northern Indiana and were studied by pure culture methods in an attempt to determine their identity. This paper describes the results of this investigation.

METHODS AND MATERIALS

Methods of study were essentially those used by Gordon and Smith (6). Modified or different procedures will be mentioned under "Results". All media were prepared from Difco products, except trypticase, manufactured by the Baltimore Biological Laboratories (BBL). The agar used was shredded bulk agar (American Agar Co.).

Certain techniques were found to give better and more consistent results than others when bacteria were studied at 65° C. Large inocula were necessary to insure growth. Consequently, agar slant media were inoculated with a bent needle, rather than with the tip of a straight needle. Agar plate media were inoculated with a loop. Liquid media were seeded by using one or two drops, or, occasionally, 0.5 ml. per 20 ml. of medium. A 1 ml. pipette was employed for the transfers. In order to lessen the effect of evaporation at 65° C., special antibiotics assay plates were used, holding 25-30 ml. of agar. Wide test tubes (150 × 25 mm.), containing 20 ml. of medium, were found to be more satisfactory than ordinary, narrow test tubes.

Unfortunately, Gordon and Smith (6) did not state at what temperature of incubation most tests were carried out. It appears[4] that all determinations were made at 45°-50° C. in order to compare *Bacillus stearothermophilus* with *Bacillus coagulans*.

---

[1] Part 1 of a thesis submitted to the graduate school of Purdue University by the senior author in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Post-doctoral Fellow.

[3] Professor of Bacteriology.

[4] Gordon, R. E., private communication. 1950.

In this investigation, 65° C. was used routinely. Tests were also performed at 50° C., in order to make results comparable to those of Gordon and Smith. Incubation at 65° C. was done in a horizontal forced draft incubator, sufficiently well insulated to keep the temperature constant. This was ascertained by placing thermometers immersed in water at various locations in the incubator. The 50° C. incubator was an ordinary incubator which also remained constant. For temperatures above 65° C. a covered water bath was used.

RESULTS

Isolation, Purification and Care of Cultures

Thirty-four amylolytic, stenothermophilic, bacterial cultures were isolated from a sample of muck soil from Northern Indiana (Frazier Farm, Akron, Indiana) on two occasions: the first group of 14 isolates in November 1948, primarily on their ability to hydrolyze the naturally occurring starch in potatoes; the second group of 20 isolates in February 1950, primarily on the basis of their ability to reduce nitrate to gaseous compounds. The latter characteristic was only rarely observed with thermophilic bacteria (5). A quantitative plate count on a medium consisting of 1 per cent trypticase, 0.5 per cent yeast extract, 0.2 per cent NaCl, 1 per cent soluble starch and 2 per cent agar, incubated for 24 hours at 65° C., showed that there were still approximately 15,000 thermophilic bacteria per gram of soil present in February 1950. The sample of soil was stored in a covered glass jar at room temperature during the 15 months' period.

The first group was isolated as follows: Ten gm. of soil were mixed with 100 ml. of sterile water in a Waring blendor. Decimal dilutions were made and three plates prepared from each dilution, using 1 ml. inoculum per plate. The medium was a modified version of the KP medium of Imshenecki, Solntzewa and Kuzyurina (7) and is described elsewhere (14). Cultures were selected which hydrolyzed the starch vigorously within 24 hours at 65° C. Zones of hydrolysis were detected by flooding the plates with Lugol's iodine solution.

The second group was isolated as follows: Again decimal dilutions were prepared from the same sample of soil used above. This time, the plating medium consisted of 1 per cent trypticase, 0.5 per cent yeast extract, 1 per cent soluble starch agar. Cultures which hydrolyzed starch and 2 per cent broth of the same composition and which formed gas from 0.1 per cent KNO_3 nutrient broth were selected.

Purification was done in cotton stoppered 6 oz. medicine bottles, using three bottles per culture. The medium consisted of 1 per cent trypticase, 0.5 per cent yeast extract, 0.5 per cent soluble starch and 2 per cent agar. Twenty-five ml. were used per bottle. One drop of inoculum (trypticase-yeast extract broth) was put into the first bottle and well mixed with the agar. One drop was then transferred from the first into the second bottle and again well mixed. Two drops were transferred into the third, and last bottle. The bottles were placed flat on the table, allowed to harden and were incubated at 65° C.

A certain mass inoculum effect was observable. The first bottle showed heavy growth after 12 hours of incubation. The third bottle,

FIGURE 1. Flagella of *B. stearothermophilus* ATCC No. 7954 Chromium shadow cast. Negative print. Magnification approx. 25,000 X.

FIGURE 2. Flagella of *B. stearothermophilus* ATCC No. 7954 Chromium shadow cast. Negative print. Magnification approx. 25,000 X.

which contained only a few colonies on the surface of the agar, required two days of incubation. Apparently, something was lacking that would yield good growth in a short time, even when small inocula were used.

The purification procedure was repeated two more times. Well isolated, single colonies of one type were selected. They were small (3-5 mm.), circular, convex, glistening, entire, smooth and colourless. One culture (No. 6) came from dull, non-glistening, flat, spreading colonies. Large irregular, flat, whitish, transparent colonies were ignored.

Stock cultures were stored on potato agar slants in the refrigerator and were transferred every six months. Between transfers the cells were grown in trypticase-yeast extract broth for 24 hours at 65° C. The use of soluble starch for this purpose is optional. For working purposes, cultures were grown and stored on trypticase-yeast extract agar slants without starch. All cultures remained viable under these conditions.

Bacillus stearothermophilus ATCC No. 7954 was obtained from the American Type Culture Collection, Washington, D.C., and was carried along as standard of comparison. This gave a total of 35 cultures.

Description of Cultures

Morphology

Cells grown on 1 per cent trypticase, 0.5 per cent yeast extract, 2 per cent agar slants were stained with 0.5 per cent aqueous safranin. Cultures 12 hours old: Straight rods, rounded ends, staining evenly, arranged singly or in short chains. Culture No. 7 formed long filaments. Cultures 96 hours old: Irregular shaped rods, containing granular material, staining unevenly, short chains and long filaments. On nutrient agar slants, cells disintegrated finally, leaving debris, granules, spores and ghost cells behind. On trypticase-yeast extract agar, lysis was delayed, because evenly staining cells were still observed after three months of storage in the refrigerator.

Cells ranged from 2.8 to 5.0 microns in length and from 0.5 to 0.7 microns in width. Sporebearing cells were always swollen. Free spores, 2.0×0.9 microns, were highly refractive and always wider than the vegetative cell. All cultures formed terminal or subterminal, oval, bulging spores.

Sporulation was not constant for all strains under a given set of conditions. Strains not sporulating at 65° C. did so readily at lower temperatures. Type of medium, temperature of incubation and location on the agar slant seemed to influence spore formation.

All cultures were motile in hanging drop preparations of 18-hour old nutrient broth cultures. Difco motility agar was less suitable for the determination of motility. Stab inoculations were made, incubated at 65° C. and were observed daily for two days. Only four cultures showed infundibuliform growth away from the stab. All other strains grew well on the surface of the medium, but not in the stab.

No flagella stain was attempted. Black and Tanner (1) reviewed briefly the futile attempts made by several investigators to stain flagella of thermophilic bacteria; mesophilic bacteria on the same slide stained well, but not the thermophiles. Peritrichous flagella demonstrated by the electron microscope are shown in Figures 1 and 2. The cells were

grown at 65° C. for 6 hours on a medium consisting of trypticase soy broth (BBL) 2 per cent, heart infusion broth (Difco) 1 per cent, yeast extract (Difco) 0.3 per cent and agar 1.5 per cent, using distilled water.

Since the Gram stain is highly variable and of little diagnostic value with this group of bacteria it was not attempted.

Temperature of Growth

Strains were cultured in trypticase-yeast extract broth or on agar slants of the same composition until growth was observed. The effect of temperature on growth, regardless of the length of incubation, is shown in Table 1.

TABLE 1.—NUMBER OF CULTURES GROWING IN TRYPTICASE YEAST EXTRACT BROTH AT VARIOUS TEMPERATURES, REGARDLESS OF THE PERIOD OF INCUBATION

Temperature of incubation ° C.							
30	37	45	50	65	70	72.5	75
0	9	30	35*	35	31	5	0

* Nine strains showed delayed start of growth.

These bacteria were stenothermophiles with an optimum temperature range from 55° to 65° C. The upper temperature range was sharply defined, because 31 cultures grew at 70° C., while only 5 grew at 72.5° C. The lower temperature was ill defined, ranging from about 37° C. to over 45° C. It was a strain characteristic, because some of the cultures which did not grow at 45° C. showed a delayed start of growth at 50° C. All cultures grew readily at 65° C., which was used routinely.

Cultural Characteristics

The cultural and biochemical characteristics of the 35 strains of stenothermophilic bacteria are presented here as briefly as possible. Greater details and certain interpretations of the results have been presented elsewhere (13).

Nutrient broth (65° C.): All cultures grew, but not particularly well. Some strains, which did not produce any turbidity, formed colonies when the clear broth was streaked on nutrient agar.

Five per cent NaCl nutrient broth (65° C.): None of the cultures grew.

Trypticase broth (65° C.): Reinforced by 0.5 per cent yeast extract or by soluble starch supported the growth of all cultures. Inocula were grown routinely in 1 per cent trypticase, 0.5 per cent yeast extract broth (using distilled water).

Proteose-peptone, neopeptone, tryptose broth (65° C.): 1.5 per cent protein plus 1 per cent soluble starch inhibited the growth of most strains. Adding 0.5 per cent yeast extract to 1 per cent tryptose- 1 per cent soluble starch broth alleviated the inhibition somewhat. Proteose-peptone broth used for the detection of acetylmethylcarbinol also inhibited certain strains constantly. The cause of this inhibition was not investigated.

Nutrient agar (65° C.): All cultures grew within a day. Growth was smooth, glistening, raised, whitish, except for three strains which were dull, flat and irregular. Two of the latter cultures tended to dissociate into smooth and rough colonies.

Trypticase yeast extract agar (65° C.): All cultures grew well within two days, many cultures forming beaded colonies along the streak after 12 hours of incubation.

Glucose agar (50° and 65° C.): All cultures grew within a day at both temperatures.

Soybean (variety Black Hawk) agar: All cultures grew within 18 hours at 65° C. At 50° C., all cultures but one grew. After 18 hours of incubation, 11 strains showed no growth, while 7 strains had only traces of growth. This delay was caused by the temperature and not by the medium.

Glucose-asparagine agar (50° and 65° C.): Certain strains failed to grow regardless of the temperature of incubation. At 65° C., seven cultures failed to grow after 24 hours, five after 3 days of incubation. At 50° C., cultures developed only slowly; after 24 hours 15 strains grew, after 48 hours 26 strains and after 3 days 30 strains grew. Again five cultures failed to develop.

Stock culture agar (Difco): None of the isolates grew at 65° C. Only two strains showed some growth after 3 days of incubation at 50° C.

Proteose-peptone acid agar, pH 5.0 (50° C. and 65° C.): None of the cultures grew after 3 days of incubation.

Citrate agar (65° C.): None of the cultures grew after 3 days of incubation.

Lead acetate agar (Difco) (65° C.): Stab cultures did not grow at all; spot inoculations on lead acetate agar plates also failed to grow.

pH range was tested in 1 per cent trypticase, 0.5 per cent yeast extract, 0.5 per cent soluble starch broth at 65° C.: *Bacillus stearothermophilus* ATCC No. 7954 was the only strain growing from pH 5.2 to pH 9.0. At pH 5.2 only three cultures grew, while at pH 9.0 four strains developed. Two of these had a lag extending over two days. All cultures grew readily at pH 6, 7 and 8.

Aerobiosis was tested in trypticase-yeast extract broth contained in long, narrow test tubes (300 × 18 mm.), the depth of the medium being 22 cm. At 65° C., all but four cultures showed uniform turbidity throughout the medium. The 4 strains formed a pellicle leaving the medium clear.

Biochemical Characteristics

Hydrolysis of Gelatin: (*a*) By the method of Gordon and Smith—13 strains cleared the whole plate, 9 strains showed a wide zone of hydrolysis, 10 strains showed a narrow zone of hydrolysis (including *B. stearothermophilus* ATCC No. 7954) and 3 strains gave no zone at all after 2 days of incubation at 65° C.

(*b*) By using trypticase-yeast extract—12.8 per cent gelatin in tubes, incubating them for 6 days at 50° C. and at 65° C., respectively. The tubes were checked for liquefaction by placing them into the refrigerator for 3 hours after 3 and 6 days. All cultures grew at both temperatures. At 50° C., 24 cultures hydrolyzed gelatin; at 65° C., 33 cultures attacked the substrates. Since only two strains failed to hydrolyze gelatin at both temperatures, better gelatinase activity was obtained at the higher temperature.

Litmus Milk: (*a*) Tomato juice-yeast extract-litmus skim milk at 65° C.—all cultures reduced the milk and formed a soft curd after 3 days of incubation. The controls remained unchanged for 4 days, but deteriorated on prolonged incubation.

(*b*) Plain litmus skim milk at 50° C. for 2 weeks—only 9 strains did not attack the milk. The other 26 cultures reduced the milk and formed a soft curd.

(*c*) Plain litmus skim milk at 65° C. for 4 days—32 cultures reduced the milk and formed a soft curd. Three strains left the milk unchanged.

Depending on the cultural conditions (composition of the milk, temperature and length of incubation), all strains reduced the milk and formed a soft curd.

Reduction of Nitrate (50° C. and 65° C.): Thirty strains reduced 0.1 per cent nitrate to nitrite at both temperatures. Five cultures failed to do this. The presence of nitrate in these culture media could be demonstrated after 6 days of incubation. *B. stearothermophillus* ATCC No. 7954 reduced nitrate to nitrite, but not further.

Reduction of Nitrite (65° C.) after 24 hours of incubation was indicated by the formation of a gas by 24 strains. Twenty of these cultures were isolated originally on the basis of their ability to form gas in KNO_3 broth. The gas was not identified.

Formation of Acetylmethylcarbinol (65° C.) was tested after 1, 3 and 5 days of incubation: All cultures were negative. Some strains were inhibited by the proteose-peptone of the medium.

Formation of Indol (65° C.): When tested with Kovac's reagent after 2 and 4 days of incubation, all cultures were negative in 1.5 per cent tryptone broth. In 1 per cent tryptone broth, tested after ½, 2 and 3 days, again all strains were negative.

Formation of Dextran (50° C. and 65° C.): Was tested in the medium of Niven, Kiziuta, and White (9), incubating cultures for 6 days. No increase in viscosity was noticeable, although growth was excellent in this medium.

Formation of Hydrogen Sulphide: All cultures formed H_2S from tryptone broth depending on the cultural conditions. These are shown in Table 2.

TABLE 2.—NUMBER OF H_2S POSITIVE CULTURES UNDER VARIOUS CULTURAL CONDITIONS

Per cent tryptone broth	50° C.			65° C.		
	Days of incubation					
	3	6	Per cent increase	3	6	Per cent increase
1.0	19.0	27.0	22.8	13.0	18.0	14.3
1.5	24.0	28.0	11.4	27.0	28.0	2.9
Per cent increase	14.3	2.9	—	11.4	28.6	—

It is not possible to draw extensive conclusions from the limited number of data presented in the above tabulation. However, 1.5 per cent tryptone at 65° C. yielded the same number of H_2S forming cultures after 3 days of incubation as was obtained in 1 per cent tryptone broth after 6 days at 50° C. This seems to indicate that the enzymes involved acted faster at 65° C. than at 50° C.

A concentration of 2 per cent tryptone was used to determine whether certain, doubtful cultures might not benefit from a greater substrate concentration. This was actually the case with 6 cultures at 50° C. and with 4 cultures at 65° C.

Length and temperature of incubation, substrate concentration and strains used influenced the results.

Hydrolysis of Starch: All cultures hydrolyzed starch under a variety of cultural conditions. Since the ability of these strains to form amylase was studied in great detail it was made the subject matter of a separate paper (14).

Fermentation of Carbohydrates: The method of Gordon and Smith (6) was applied as follows: Loop inoculations of 20-hour old trypticase-yeast extract broth cultures were placed on nutrient agar containing 0.5 per cent of the carbohydrate to be tested and brom cresol purple as indicator. The formation of acid was checked after 18 hours and 42 hours of incubation at 65° C. One set of plates, including five different carbohydrates, was prepared at one time and inoculated with the same inoculum. The entire experiment was repeated at a later date, following the same technique. Results are summarized in Table 3.

TABLE 3.—NUMBER OF CULTURES SHOWING ACID FORMATION ON CARBOHYDRATE NUTRIENT AGAR AFTER 18 AND 42 HOURS OF INCUBATION AT 65° C.

Carbohydrate tested	Replication				Average	
	a		*b*			
	Hours of incubation					
	18	42	18	42	18	42
Arabinose	22	13	20	6	21.0	9.5
Xylose	21	11	12	6	16.5	8.5
Glucose	34	31	33	18	33.5	24.5
Maltose	29	31	33	10	31.5	20.5
Dextrin	20	11	10	3	15.0	7.0

The striking feature of this experiment was the large number of cultures which changed from an acid to an alkaline reaction within 42 hours of incubation.

While these experiments were in progress, Vera (15) presented evidence showing that commercial peptones and beef extracts contained enough fermentable carbohydrate material to yield acid or acid and gas when inoculated with *Escherichia coli*. The following media were prepared in 2 per cent concentration, adjusted to pH 7.4 and inoculated with *E. coli*: peptone, protone, tryptone, tryptose, proteose-peptone, neopeptone, yeast extract, which were Difco products, and trypticase, a BBL product. Phenol red was used as indicator. Results are shown in Table 4.

TABLE 4.—VISUAL AND pH CHANGES OF VARIOUS SOLUBLE PROTEIN SOLUTIONS INOCULATED WITH *E. coli* AT 37° C.

Medium	Hours incubated				pH after 48 hours	
	24		48			
	Acid	Gas	Acid	Gas	Sample	Control
Peptone	+++	−	−	−	7.7	7.4
Protone	−	−	−	−	7.5	7.4
Tryptone	+	−	−	−	7.4	7.4
Tryptose	++	−	−	−	7.4	7.4
Trypticase	−	−	−	−	8.0	7.4
Proteose peptone	−	−	−	−	7.5	7.4
Neopeptone	−	−	−	−	7.6	7.4
Yeast extract	+	−	−	−	7.6	7.4

Acid appeared after 24 hours of incubation in peptone, tryptone, tryptose and yeast extract as shown by the colour changes of the indicator. The acid reaction disappeared during the next 24-hour interval. Consequently, these four media were retested using glass electrode measurements after 24 and 48 hours of incubation for greater accuracy. The results of this test are shown in Table 5.

TABLE 5.—HYDROGEN ION CONCENTRATION OF FOUR PROTEIN MEDIA INOCULATED WITH *E. coli* AT 37° C. AFTER 24 AND 48 HOURS OF INCUBATION

Medium	Hours incubated		Control
	24	48	
Peptone	7.3	7.9	7.6
Tryptone	7.4	7.6	7.6
Tryptose	7.2	7.8	7.6
Yeast extract	7.0	7.6	7.6

Again, acid formation took place within the first 24 hours of incubation, but disappeared during the next 24-hour interval.

These findings explain in part the results obtained with carbohydrate nutrient agar plates. It is impossible to state whether the acid formed came from the sugar under test or from the fermentable carbohydrates of the peptone used to prepare the nutrient agar. Bozeman and Orcutt (3) and Orcutt, Bozeman and Coffee (10) stressed the fact that acid formation from carbohydrates by sporebearing bacteria was a doubtful criterion of utilization.

TABLE 6.—COMPARISON OF DESCRIPTIONS OF STRAINS OF BACTERIA APPARENTLY BELONGING TO *Bacillus stearothermophilus*

Characters	Donk, 1920	Gordon and Smith, 1949	Prickett, 1928	Stark and Tetrault, 1951
Number of strains	1	87	7	35
Source	Canned corn	Soil, milk, food, silage	Milk	Soil
Vegetative cells	Rods	Rods	Rods	Rods
Size in μ	3.5 × 0.8	2.5-3 × 0.9-1	2-4.5 × 0.7	2.5-5 × 0.5-0.7
Location of spores	Terminal	Terminal, subterm.	Term., subterm.	Term., subterm.
Size in μ	1 × 1.5	1-1.2 × 1.5-2.2	0.4-0.5 × 0.6-0.8	0.9 × 2
Sporangium	—	Bulging	Bulging	Bulging
Motility	Non motile	Motile	Motile	Motile
Flagella	None	—	—	Peritrichous
Gram stain	Negative	Variable	Positive	—
Aerobiosis	Facul. anaerob.	Facult. anaerob.	Facul. anaerob.	Facul. anaerob.
Nutrient broth	Clouded	—	Slight clouding	Clouded
Pellicle	None	—	None	None
Sediment	Compact	—	None	Viscid
Nutrient agar slants	Beaded, filif.	Beaded, filif.	Beaded, filif.	Beaded, filif.
Colonies	Circular, 2 mm.	Circular or irregular	Circular, 3 mm.	Circular, 3 mm.
Litmus milk, plain	Reduced, pept.	—	Rennet coagulum, 63° C.	91% coag. at 65° C.; 74% coag. at 50° C.
Litmus milk, tomato juice	—	Not coagulated* at 50° C.	—	100% coag. at 65° C.
Gelatin liquefaction	Negative	Positive, 0.4%, 50° C.	Positive, 10%*, 56° C.	Positive, 12.8%*, 65° C.
Hydrolysis of starch	Positive	Positive	Positive	Positive
Formation of H_2S	—	—	Doubtful	Positive

* Substrate concentration.

TABLE 6.—COMPARISON OF DESCRIPTIONS OF STRAINS OF BACTERIA APPARENTLY BELONGING TO *Bacillus stearothermophilus*—*Continued*

Characters	Donk, 1920	Gordon and Smith, 1949	Prickett, 1928	Stark and Tetrault, 1951
Indole formation	Negative	—	Negative	Negative
Acetoin formation	—	Negative	Negative	Negative
Nitrate reduction	Negative	88% positive	Positive	86% positive
" " to gas	—	—	Positive	69% positive
Ammonia formation	Negative	—	Positive	—
Proteose peptone agar	—	Negative	—	Negative
Soybean agar	—	98% negative	—	100% positive
Glucose agar	—	89% negative	—	100% positive
Glucose asparagine agar	—	49% negative	—	14% negative
Stock culture agar	—	93% negative	—	100% negative
Citrate agar	—	97% negative	—	100% negative
Five per cent NaCl broth	—	Negative	—	Negative
Glucose	Acid	100% acid	Acid	94% acid
Xylose	—	45% acid	No growth	Acid
Arabinose	—	24% acid	—	Acid
Maltose	—	—	No change	Acid
Lactose	Acid	—	No change	—
Sucrose	Acid	—	Alkaline	—
Potato slice	No growth	—	Variable growth	—
Optimum temperature	50° C.	50° C.-60° C.	60° C.-65° C.	55° C.-65° C.
Minimum temperature	45° C.	84% positive at 37° C.	About 37° C.	About 37° C.
Maximum temperature	76° C.	51% positive at 70° C.	73° C.-75° C.	About 71° C.

DISCUSSION

Prickett (11) described seven strains of *Bacillus kaustophilus* in great detail. This species was found by Gordon and Smith (6) to belong to *Bacillus stearothermophilus*. Consequently, the description of *B. kaustophilus* Prickett, of *B. stearothermophilus* Donk and of *B. stearothermophilus* Donk as emended by Gordon and Smith will be compared with that obtained in this investigation. The results are summarized in Table 6. It may be noted that the description of *B. kaustophilus* agrees more closely with the strains under study than with the emended description by Gordon and Smith.

Gordon and Smith established a species pattern for *Bacillus stearothermophilus* and defined it: "Identification of a strain was not intended to depend on one or two, but rather on a group of characters or on a species pattern. If the reactions and properties of a strain conformed in the main, not necessarily in every detail, to a species pattern, the strain was considered a member of the species." Gordon* stated again concisely the major and minor characters of the species. The more important characteristics were:

1. The mature spore being wider than the 24-hour old vegetative rod.
2. Growth at 65° C. waterbath temperature.
3. No growth on proteose peptone acid agar.
4. Wide zone of hydrolysis on gelatin plates.
5. No curd in tomato yeast milk after 3 days at 45° C. or 50° C.
6. Hydrolysis of starch.
7. Failure to form acetoin.
8. Lack of growth in 5 per cent NaCl nutrient broth.
9. Inhibition on soybean, glucose and stock culture agar as compared with growth on nutrient agar.

Of lesser importance were: Reduction of nitrate to nitrite, lack of growth on citrate agar, and pH less than 5.5 in glucose broth after 7 days of incubation.

It may be noted that five of the nine major characters are based on failure to do something.

B. White (16) wrote: "The strongest objection is to be made to the definition of any unit or group on negative characters." The writers are inclined to agree with this point of view. It is always more difficult to demonstrate that a bacterial culture does something than to show that it does not. This is particularly true of thermophilic bacteria where very little or no knowledge exists concerning their optimum requirements for growth and metabolic activity. Results of this study concerning the hydrolysis of starch, the hydrolysis of gelatin, the formation of H_2S, and other metabolic activities, indicated that positive findings were only obtained under a specific set of environmental conditions. Disregarding these conditions and reporting the species as being unable to do something contributes very little to a better understanding of its characteristics.

In view of the observations made in this study, supported by the description of *Bacillus kaustophilus* by Prickett, a modification of the species pattern of *Bacillus stearothermophilus* is suggested. The modified species pattern is shown in Table 7.

* Private communication. 1950.

TABLE 7.—THE SPECIES PATTERN OF *Bacillus stearothermophilus*

Gordon and Smith (1949)	Stark and Tetrault (1951)
1. Mature spores bulge cells	Same
2. Growing at 65° C.	Same
3. No growth on proteose peptone ag.	Same
4. Failure to form acetoin	Same
5. Failure to grow in 5 per cent NaCl broth	Same
6. Wide zone of hydrolysis on 0.4 per cent gelatin plates	Hydrolysis of gelatin in tubes (trypticase-yeast ex.-gelatin)
7. 1 per cent potato starch agar hydrolyzed	0.5 per cent soluble starch broth hydro.
8. No curd in tomato yeast milk, 50° C.	Curd in tomato yeast ex. milk, 65° C. Curd in plain litmus milk, 65° C.
9. Inhibition on soybean, glucose, and stock culture agar as compared with nutrient agar	Growth on soybean, glucose and nutrient agar, none on stock culture, citrate and lead acetate agar.
10. Incubated at 45° C.-50° C.	H_2S formed from 1, 1.5 or 2 per cent tryptone broth. Incubated at 65° C.

Ignoring the failures to do something, points 1, 2, 6, 7, 8, 9 and 10 determine whether or not an unknown strain belongs in this species.

SUMMARY

1. Thirty-four strains of stenothermophilic bacteria were isolated from muck soil of Northern Indiana.

2. A determinative study by the methods of Gordon and Smith was made in an attempt to describe, identify and classify the purified cultures.

3. All strains belonged to *Bacillus stearothermophilus* as defined by Gordon and Smith.

4. A modification of the species pattern as given by Gordon and Smith is proposed.

5. Bacteria growing at 65° C., forming bulging spores, hydrolyzing gelatin and starch, coagulating milk, forming H_2S in tryptone broth, growing on nutrient-, glucose and soybean agar, not growing on proteose-peptone acid agar, pH 5.0, citrate and lead acetate agar when tested at 65° C., may be considered as belonging to *Bacillus stearothermophilus*.

ACKNOWLEDGMENTS

The writers wish to thank K. S. Read, Department of Biological Sciences, Purdue University, Lafayette, Indiana, who prepared specimens for electron microscopy and photographed them. The financial assistance of the Purdue Research Foundation for carrying out the project is gratefully acknowledged.

REFERENCES

1. Black, L. A., and F. W. Tanner. A study of thermophilic bacteria from the intestinal tract. Zentralb. Bakt., Infekt., Parasitenk. Abt. II, 75 : 360-375. 1928.
2. Breed, R. S., E. G. D. Murray, and A. P. Hitchens. Bergey's manual of determinative bacteriology. 6th ed. Williams and Wilkins Co., Baltimore. 1948.
3. Bozeman, S. R., and F. S. Orcutt. Glucose utilization compared with pH production when influenced by concurrent decomposition of peptone by the genus *Bacillus*. J. Bact. 49 : 108. 1945. (*Abstract*).

REFERENCES—Continued

4. Donk, P. J. A highly resistant thermophilic organism. J. Bact. 5 : 373-374. 1920.
5. Gaughran, E. R. L. The thermophilic microorganisms. Bact. Rev. 11 : 189-225. 1947.
6. Gordon, R. E., and N. R. Smith. Aerobic sporeforming bacteria capable of growth at high temperatures. J. Bact. 58 : 327-341. 1949.
7. Imshenecki, A. A., L. I. Solntzewa, and L. A. Kuzyurina. Thermophilic amylolytic bacteria. Mikrobiologiya 11 : 29-45. 1942.
8. Knight, B. C. J. G., and H. A. Proom. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. J. Gen. Microbiol. 4 : 508-538. 1950.
9. Niven, C. F., Z. Kiziuta, and J. C. White. Synthesis of a polysaccharide from sucrose by *Streptococcus* S. E. B. J. Bact. 51 : 711-716. 1946.
10. Orcutt, F. S., S. R. Bozeman, and W. B. Coffee. Further evidence demonstrating the utilization of glucose, sucrose and d- and l-arabinose by certain members of the genus *Bacillus* in which the pH of the sugar peptone medium is not appreciably lowered. J. Bact. 49 : 109. 1945.
11. Prickett, P. S. Thermophilic and thermoduric organisms with special reference to species isolated from milk: V. Description of sporeforming types. N.Y. State Agr. Exp. Sta. Tech. Bull. 147. 1927.
12. Smith, N. R., R. E. Gordon, and F. E. Clark. Aerobic mesophilic sporeforming bacteria. U.S.D.A. Misc. Publ. 559. 1946.
13. Stark, E. Stenothermophilic bacteria and their starch splitting enzymes. Ph.D. thesis, Purdue University. 1951.
14. Stark, E., and P. A. Tetrault. Observations on amylolytic bacteria. III. Cultural conditions necessary for the formation of amylase by thermophilic bacteria of the *Bacillus stearothermophilus* group. *In preparation.* (Abstract in Bact. Proc., p. 27. 1951).
15. Vera, H. D. Relation of peptones and other culture media ingredients to the accuracy of fermentation tests. Am. J. Publ. Health 40 : 1267-1271. 1950.
16. White, B. P. Remarks on bacterial taxonomy. Zentralb. Bakt., Infekt., Parasitenk. Abt. II. 96 : 145-149. 1937.

NARCISSUS BULB FLY CONTROL WITH METHYL BROMIDE FUMIGATION AND ITS EFFECT ON THE FLOWER PRODUCTION OF GREENHOUSE-GROWN NARCISSUS BULBS[1]

H. Andison and W. T. Cram[2]

Dominion Entomological Laboratory, Victoria, B.C.

[Received for publication June 14, 1951]

INTRODUCTION

Since its discovery as a fumigant by Le Goupil in 1932, methyl bromide has become one of the most common fumigants. The history of this fumigant has been thoroughly reviewed by Mackie and Carter (11) and Mackie (12), who pioneered its use on this continent. Much work has been done and many papers have been published on the dosage of methyl bromide required for the control of stored product insects and on the effect of the fumigant on nursery stock.

The use of methyl bromide for atmospheric fumigation of narcissus bulbs to control the larvae of the narcissus bulb fly, *Lampetia equestris* (F.), was first reported by Blanton (1). He fumigated non-infested King Alfred bulbs at 2.5 and 3 pounds of fumigant per 1,000 cubic feet for 4 hours at 70° F. to test their tolerance to this treatment. Examination of the flowers the following spring indicated that the treatments had no adverse effect on the bulbs, that there was no apparent injury to the flowers, and that at the time of digging there was no significant difference between the weights of treated and untreated bulbs. As a result of these experiments a recommendation of 3 pounds of methyl bromide per 1000 cubic feet for 4 hours at 70° F. was suggested by Blanton for the control of the narcissus bulb fly infesting the King Alfred variety. Essentially the same results were obtained by Mackie, Steinweden, and Carter (13) in experiments to control the narcissus bulb fly and the bulb mite, *Rhizoglyphus echinopus* (F. & R.).

The tolerance of narcissus bulbs to methyl bromide fumigation was further reported on by Breakey (3). King Alfred bulbs were fumigated at rates up to 3 pounds per 1000 cubic feet for 4 hours at 70° F. Field-grown bulbs did not show any injurious effects from the treatment.

The main limiting factor of this method of fumigation, which during the last five years has been practised by most bulb growers in California, Oregon, and Washington, is the high temperature of 70° F. This necessitated the installation of expensive electrical heating equipment in the fumigation chambers of the Pacific Northwest. Examination of the literature indicated that no work had been done to determine the degree of safety of this fumigant to the main narcissus varieties at a temperature of 60° F. with dosages giving 100 per cent mortality of narcissus bulb fly larvae. Also, information appeared to be lacking on the effect of methyl bromide fumigation on the flower production of greenhouse-forced bulbs.

The experiments described in this paper were conducted during 1949 and 1950 to determine: (*a*) an effective dosage of methyl bromide at a narcissus bulb temperature of 60° F. (the monthly mean temperature at

[1] Contribution No. 2839, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] Agricultural Research Officer and Technical Officer, Dominion Entomological Laboratory, Victoria, B.C.

digging time), which would obviate the installation of expensive heating equipment in the fumigation chamber; and (*b*) the effects of various standard and excessive dosages of methyl bromide on the flower production of the main varieties of narcissus bulbs grown and forced in British Columbia.

MATERIALS AND METHODS

With the co-operation of the Department of Agriculture of British Columbia, an experimental fumigation chamber, 25 × 29 × 48 inches, was built. The chamber was constructed of 7-ply plywood, and all joints were sealed on the inside with asphalt and quarter-round moulding. The inside of the chamber was painted with two coats of ethyl cellulose paint. The door was the full size of the end of the chamber and was attached by means of three heavy hinges at the top so that it could swing outward and upward when opened. The door was clamped shut by two straps of iron, which projected on either side and were secured to the chamber by means of wing nuts. Both felt and sponge-rubber gaskets sealed the door to the chamber. A small gas-tight window, 4 × 7 inches, was fitted in the door. A false floor and a small electric fan were placed inside the chamber. During the 1949 experiments the chamber was not equipped with any means of temperature control; experiments were conducted at the prevailing atmospheric temperatures. In 1950 the chamber was insulated with 8 inches of fir sawdust and fitted with a manually controlled set of four 100-watt electric light globes. Also in 1950 the chamber was fitted with a 280-cc. applicator for releasing the methyl bromide in 2-cc. amounts. The amount of fumigant released in the chamber corresponded to the stated dosage of fumigant in pounds per 1000 cubic feet.

Infested King Alfred narcissus bulbs were used for mortality counts in all experiments. Infestation was determined by scraping the basal plate of the bulb with a sharp knife and observing the small, round larval entrance hole in the root ring; the hole was usually surrounded by dark-brown, decayed bulb tissue. Larvae were not found in all the bulbs showing entrance holes, as there is a high natural mortality of the larvae in the bulbs after harvest.

All fumigation treatments were replicated three times. Each replicate comprised 33 bulbs, all of which were placed in the chamber; of these 8 were not infested and were to be used in greenhouse forcing tests. Hence each treatment had a total of 75 infested bulbs for mortality counts and 24 non-infested bulbs for forcing tests. The forcing stock in 1949 consisted of No. 1 double-nose King Alfred bulbs (18-20 cm. in circumference), and in 1950 of No. 1 single-nose King Alfred bulbs (15-18 cm. in circumference) along with No. 1 double-nose Rembrandt, Golden Harvest, and Magnificence bulbs. The grades cited are established by the Destructive Insect and Pest Act in effect on and after April 1, 1949.

Bulbs were placed in the fumigation chamber and allowed to reach the desired temperature before the fumigant was metered into the chamber. Usually it took about 1 hour to raise the bulb temperature from 60° F. to 70° F., when the chamber temperature was 70° F. The temperature in the chamber was recorded by the use of an ordinary chemist's thermometer registering 0-212° F., the mercury end being thrust into the centre of an

infested bulb. The thermometer was hung inside the chamber in such a way that it could be read through the glass window in the door. The relative humidity inside the chamber during the bulb fumigation was very constant at 70 to 75 per cent. The electric fan was run for the first 15 minutes and for 15 minutes each following hour, except in one treatment in 1950 when the use of the fan was purposely omitted. The schedules selected for the fumigation experiments appear in Tables 1, 2, and 3.

The infested bulbs were checked for larval mortality no sooner than 48 hours after fumigation by cutting the bulbs open, removing the larvae, and placing them in water in a Petri dish. Movement, if any, was observed when the mouth hooks of the larvae were probed. The bulbs for the greenhouse forcing studies were planted on October 5, 1949, and on September 27, 1950. Each replicate of 8 bulbs was planted in a wooden flat measuring 12 × 6 × 5 inches. The soil was a mixture of sand, peat-moss, and compost in equal quantities (by volume). The flats were placed outside and covered with 4 inches of sand, and the bulbs were left to develop

TABLE 1.—AVERAGE MORTALITIES OF NARCISSUS BULB FLY LARVAE AND FLOWER STEM LENGTHS OF NON-INFESTED KING ALFRED NARCISSUS BULBS FUMIGATED WITH METHYL BROMIDE AT VARIOUS RATES AND EXPOSURES, 1949

Methyl bromide lb./1000 cu. ft.	Fumigation period, in hours	Temperature of bulbs, ° F.	Number of larvae	Average size of larvae, mm.	Mortality per cent	Average flower stem length, inches
3	4	70	42	7.5	100	18.1
3	4½	60	44	7.6	100	17.4
3	8	70	42	10.5	100	17.0
3	8½	60	47	10.1	100	17.6
5	2	70	44	10.7	100	18.1
5	2½	60	48	10.2	100	17.4
Check (untreated)		—	—	—	—	17.0

TABLE 2.—AVERAGE MORTALITIES OF NARCISSUS BULB FLY LARVAE AND FLOWER STEM LENGTHS OF NON-INFESTED KING ALFRED NARCISSUS BULBS FUMIGATED WITH METHYL BROMIDE AT VARIOUS RATES AND EXPOSURES, 1950

Methyl bromide lb./1000 cu. ft.	Fumigation period, in hours	Temperature of bulbs, ° F.	Number of larvae	Average size of larvae, mm.	Mortality per cent	Average flower stem length, inches
6	1	60	27	5.8	40.0	15.5
3	4	55	30	8.7	85.3	15.1
3	4	60	31	5.8	90.0	15.3
3[1]	4	60	35	6.7	86.6	15.3
3[2]	4½	60	29	7.6	100.0	14.9
3	4	70	17	5.4	100.0	15.5
3	4	80	31	7.5	100.0	15.7
5	2	60	27	5.1	100.0	14.1
3	12	60	30	8.2	100.0	15.1
3	24	60	30	8.3	100.0	No bloom
Check (untreated)		—	—	—	—	15.3

[1] Fan not operated during fumigation.
[2] Dosage repeated after 2 days.

TABLE 3.—AVERAGE FLOWER STEM LENGTHS OF FOUR VARIETIES OF NON-INFESTED NARCISSUS AFTER FUMIGATION OF BULBS WITH METHYL BROMIDE, 1950

Methyl bromide, lb./1000 cu. ft.	Exposure hours	Temperature of bulbs, °F.	Variety	Average flower stem inches
3	4	60	King Alfred	15.3
3	8	60	King Alfred	15.2
Check	(Untreated)	—	King Alfred	15.3
3	4	60	Rembrandt	12.7
3	8	60	Rembrandt	12.1
Check	(Untreated)	—	Rembrandt	13.0
3	4	60	Golden Harvest	13.6
3	8	60	Golden Harvest	13.8
Check	(Untreated)	—	Golden Harvest	14.9
3	4	60	Magnificence	14.3
3	8	60	Magnificence	13.6
Check	(Untreated)	—	Magnificence	15.5

roots. Bulbs fumigated in 1949 and 1950 were taken into the greenhouse on January 9, 1950, and February 12, 1951, respectively. The greenhouse forcing temperature was maintained at 60° to 65° F. Records were kept of blooming dates, and blooms were cut daily. Measurements were taken of flower stem length and of perianth diameter. As there are no standard grades for the narcissus cut-flower trade, the criterion for flower production in these experiments was based on the work and experience of J. H. Crossley, Bulb Specialist, Dominion Experimental Station, Saanichton, B.C. For experimental purposes he has concluded that cull flowers have a stem length less than 10 inches and a perianth diameter less than 3 inches.

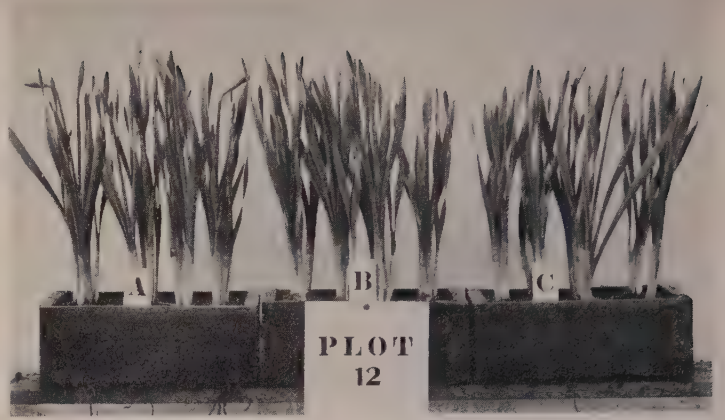
RESULTS

Results of larval mortality counts and bulb forcing studies with the King Alfred variety during 1949 and 1950 appear in Tables 1 and 2. Results of forcing studies with the varieties Rembrandt, Golden Harvest, and Magnificence during 1950 appear in Table 3.

In 1949, 100 per cent mortality of the bulb fly larvae was obtained with all of the methyl bromide treatments, i.e., 3 pounds for $4\frac{1}{2}$ or $8\frac{1}{2}$ hours at 60° F., 3 pounds for 4 or 8 hours at 70° F., and 5 pounds for 2 and $2\frac{1}{2}$ hours at 70° F. and 60° F. The non-infested King Alfred stock that was forced after these fumigations showed no significant differences in flower production from the untreated bulbs. There was no difference greater than one day between the average blooming dates of the checks and the treated material. Average flower stem length in the treated bulbs ranged from 17.0 to 18.1 inches and was 17.0 inches in the untreated stock (Table 1). All flower perianth diameters were greater than $3\frac{3}{4}$ inches. Hence, bulb development did not appear to be affected by these dosages.

Of the various treatments tested during 1950, those that gave 100 per cent mortality of the narcissus bulb fly larvae were: 3 pounds for 12 or 24 hours at 60° F., 5 pounds for 2 hours at 60° F., and 3 pounds for 4 hours at either 70° or 80° F. The necessity for higher dosages when temperature

PLOT 1. Check, untreated.



PLOT 12. Treated with methyl bromide at 3 lb. for 4½ hr. at 60° F. Fumigated twice at an interval of 2 days.

Plot 8. Treated with methyl bromide at 3 lb. for 12 hr. at 60° F.

PLOT 9. Treated with methyl bromide at 3 lb. for 24 hr. at 60° F.

FIGURE 1. Comparison of foliage and flower growth of non-infested narcissus bulbs treated with methyl bromide at varying dosages at 60° F.

were decreased was indicated where the treatment of 3 pounds for 4 hours at 55° F. and 60° F. gave only 85 and 90 per cent mortality, respectively. It appears necessary to increase the exposure period from 4 to 4½ hours to obtain maximum mortality of the larvae at 60° F. A brief treatment of 6 pounds for 1 hour at 60° F. was very unsatisfactory, the larval mortality averaging 40 per cent. The importance of good circulation of air was indicated by the mortality rate of 86 per cent when the electric fan was not used in a treatment of 3 pounds for 4 hours at 60° F.

The results of forcing tests in 1950 showed that treatments up to and including the high dosage of 3 pounds for 12 hours at 60° F. had no apparent effect on the flower production of No. 1 single-nose King Alfred bulbs. Even those bulbs that were fumigated twice with an interval of 2 days, at 3 pounds for 4½ hours at 60° F., showed no apparent differences in growth. The flowering dates were the same for all of the plots. The average flower stem lengths for these treated plots ranged from 14.1 to 15.5 inches; the untreated bulbs showed an average stem length of 15.3 inches (Table 2). All flower perianth diameters were greater than 3 inches. The bulbs fumigated with 3 pounds for 24 hours at 60° F. were almost completely ruined; only 3 of the 24 developed foliage, which was stunted, and none produced flower stems (Figure 1).

The varieties Rembrandt, Golden Harvest, and Magnificence showed no apparent differences in flower stem length and perianth diameter between untreated bulbs and those fumigated at 3 pounds for 4 or 8 hours at 60° F. For each variety, flowers bloomed within one day of the untreated stock.

SUMMARY

During 1949 and 1950 experiments were conducted with methyl bromide fumigation on the control of narcissus bulb fly larvae and on the effect of the fumigant on the flower production of greenhouse-forced narcissus bulbs. There is a wide margin of safety between methyl bromide dosages that give complete mortality of the larvae and those dosages that injure the bulbs. The dosage of 3 pounds for 1000 cubic feet for 4½ hours at 60° F. (the mean monthly temperature at digging time) gave as high mortality of the larvae as the standard treatment of 3 pounds for 4 hours at 70° F., recommended in California, Oregon, and Washington. Hence the installation of expensive electrical heating equipment, which is essential to maintain a temperature of 70° F. in bulb fumigation chambers in the Pacific Northwest, is not necessary for satisfactory control of bulb fly larvae. Differences in flower production were not apparent between untreated King Afred bulbs and those treated at the excessive dosages of 5 pounds for 2½ hours and 3 pounds for 8 or 12 hours at 60° F. Also, no differences were observed when the varieties Rembrandt, Golden Harvest, and Magnificence were treated at the rate of 3 pounds for 8 hours at 60° F. King Alfred bulbs that were fumigated twice with an interval of 2 days at 3 pounds for 4½ hours at 60° F. showed no apparent differences in growth. The flowering dates were the same for treated and untreated bulbs at the various rates. A treatment of 3 pounds for 24 hours at 60° F. rendered King Alfred bulbs useless for flower production.

ACKNOWLEDGMENTS

Some of the bulb examinations for larval mortality were done by A. P. Randall, N. V. Tonks, and B. D. Ainscough of the Division of Entomology, Victoria, B.C., and to these the authors' thanks are due. Acknowledgment is also due to J. H. Crossley, S. Arrowsmith, and D. Polson, of the Dominion Experimental Station, Saanichton; and to A. E. Richman, greenhouse operator at the University of British Columbia, for their invaluable assistance in growing and keeping daily records of the forced blooms. The writers also wish to thank the following growers who supplied bulbs for the experiments: R. Wooldridge, W. Mattick, Joe and Bert Everett, J. Thompson, C. R. Wilson, G. White, H. Paver, R. H. Richardson. D. Killen, and J. W. Howroyd.

REFERENCES

1. Blanton, F. S. Methyl bromide fumigation for the control of the narcissus bulb fly. J. Econ. Ent. 35 : 239-241. 1942.
2. Breakey, E. P. Biology and control of the bulb mites *Rhizoglyphus hyacinthi* Bvd. and *Tarsonemus laticeps* Halb. *In* Washington Agr. Expt. Sta. Bull. 435 (53rd Ann. Rept.), pp. 120-121. 1943.
3. Breakey, E. P. The effect of methyl bromide fumigation on the subsequent development of the croft lily. J. Econ. Ent. 37 : 277-279. 1944.
4. Doucette, C. F., L. Randall, C. H. Martin, R. Schopp, and P. M. Eide. Biology of the narcissus bulb fly in the Pacific Northwest. U.S.D.A. Tech. Bull. 809. 1942.
5. English, L. L. Fumigation of camellias and azaleas with methyl bromide. J. Econ. Ent. 36 : 737-743. 1943.
6. English, L. L., and G. F. Turnipseed. The fumigation of camellia and azalea cuttings with methyl bromide. J. Econ. Ent. 37 : 81-84. 1944.
7. English, L. L. A slide rule for estimating schedules for fumigation with methyl bromide. J. Econ. Ent. 37 : 114-115. 1944.
8. Fisk, F. W., and H. H. Shepard. Laboratory studies of methyl bromide as an insect fumigant. J. Econ. Ent. 31 : 79-84. 1938.
9. Hamilton, C. C. Toxicity of methyl bromide to the common red spider and to greenhouse roses. J. Econ. Ent. 34 : 232-237. 1941.
10. Livingstone, E. M., and G. R. Swank. Methyl bromide as a fumigant for pests of ornamental plants. J. Econ. Ent. 34 : 75-76. 1941.
11. Mackie, D. B., and W. B. Carter. Methyl bromide as a fumigant, a preliminary report. California Dept. Agr. Bull. 26 : 153-162. 1937.
12. Mackie, D. B. Methyl bromide—its expectancy as a fumigant. J. Econ. Ent. 31 : 70-79. 1938.
13. Mackie, D. B., J. B. Steinweden, and W. B. Carter. Methyl bromide fumigation of narcissus bulbs for the control of bulb flies and bulb mites. California Dept. Agr., Nursery and Entomological Services, mimeographed pamphlet. 1942.
14. May and Baker Limited. Fumigation with methyl bromide. Dagenham, England. 1948.
15. Monro, H. A. U. Low temperature fumigation. Canadian Ent. 77 : 193-196. 1945.
16. Steinweden, J. B. Methyl bromide fumigation. California Dept. Agr. Bull. 34, pp. 4-14. 1945.

STUDIES ON THE HOLOCELLULOSE FRACTION OF HARDWOOD LEAVES[1]

B. B. Coldwell[2] and W. A. DeLong[3]

Department of Chemistry, Macdonald College, Que.

[Received for publication June 26, 1951]

INTRODUCTION

Theoretically, the holocellulose fraction of extractive-free plant materials comprises the entire polysaccharide content of the cell-wall. The preparation of holocellulose involves the removal of soluble and adsorbed substances of non-polysaccharide nature from plant tissues by extraction with suitable inert solvents, and delignification of the extractive-free residue. The first separation of such a fraction was made 30 years ago by Schmidt and Graumann (30), who quantitatively delignified wood with chlorine dioxide and sodium sulphite solution.

Various modifications of the original procedure have been proposed (32, 33, 31, 40, 41). The official method (tentative standard) now used for determining holocellulose in wood involves the alternate treatment of extractive-free sawdust with gaseous chlorine and alcoholic monoethanolamine solution (37).

A new technique based on delignification by acid sodium chlorite was developed by Jayme (16) in 1942 and has been simplified and improved by Wise and co-workers (43, 44). Unlike the earlier procedures (19), this method can be used for preparing large samples of holocellulose from woody and non-woody plants (5, 2, 34).

The objectives of the present work were: (*a*) to determine whether large samples of chlorite holocellulose could be prepared from fresh and partially decomposed leaves of different species; (*b*) to characterize the lignin-like material in the leaf tissue and that retained in the corresponding holocellulose fractions, and (*c*) to examine the hydrolytic products of the holocellulose fractions to obtain further information (12) respecting the nature of the decomposition processes. Research on the latter phase is still in progress.

MATERIALS AND METHODS

Mature, freshly-fallen, and partially decomposed leaves of the species, American beech (*Fagus grandifolia* Ehrh.), gray birch (*Betula populifolia* Marsh.), sugar maple (*Acer saccharum* Marsh.) and poplar (*Populus grandidentata* Michx. and *P. tremuloides* Michx.) were used. The sampling areas and the methods of collection and exposure of the leaves have been described previously (12).

Representative samples of the materials were air-dried in the laboratory and ground in a Wiley mill to pass a 20-mesh screen. Subsequent analyses were made on sub-samples of the ground material. Moisture was calculated as the loss in weight when two-gram samples were dried to constant weight in an electric oven at 105° C.; ash was found by igniting weighed samples

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Que., and the Dominion Experimental Farms Service. Macdonald College Journal Series No. 288.

[2] Formerly Lecturer in Chemistry; now Chemist, Crime Detection Laboratory, R.C.M. Police, Ottawa, Canada.

[3] Associate Professor of Chemistry.

in porcelain crucibles in a muffle at 600° C. Protein was calculated as nitrogen × 6.25, nitrogen being determined by the micro-Kjeldahl method (24). The preparation of extractive-free material has been described in a previous publication (12).

Lignin was determined by the 72 per cent sulphuric acid method (28) following pretreatment with 1 per cent hydrochloric acid (24).

Methoxyl was determined by the Vieböch and Schwappach modification of the Ziesel method as described by Clark (11) and accepted with slight changes as a tentative method by the A.O.A.C. (24). The air-dried samples were weighed out in gelatin capsules (size No. 1, Eli Lilly and Company) and a suspension of red phosphorus was used in the scrubber. These modifications were recommended by Samsel and McHard (29). Furfural was estimated photocolorimetrically by the Adams and Castagne (1) modification of the Stillings and Browning (36) method. Transmittance measurements were taken with a Coleman Universal Spectrophotometer model 11, employing a purple filter with the wavelength dial set at 5180 Å. Uronic acid anhydride was determined by the procedure of Dickson, Otterson and Link (13); alpha-cellulose by mercerization with 17.5 per cent sodium hydroxide as described by Ritter (27).

The method employed for the estimation of acetyl was essentially the Lemieux (18) semi-micro adaptation of the Genung and Mallatt (14) modified Ost distillation technique.

RESULTS AND DISCUSSION

Preparation of Leaf Holocellulose

Holocellulose was isolated according to the method of Jayme (16) as modified by Wise and co-workers (44). Two grams of air-dried, extractive-free leaves were weighed into a 400 ml. beaker, 100 ml. of distilled water were added and the beaker then placed in a water bath held at 75 ± 0.5° C, as recommended by Adams and Castagne (2). When the beaker contents reached the bath temperature, 0.12 ml. of glacial acetic acid, followed by 1.5 gm. of sodium chlorite (technical grade) were added. The beaker was covered with a watch glass and the contents stirred occasionally. At the end of one hour, without cooling, the same amounts of reagents were again added, the suspension thoroughly mixed and the heating continued. The treatment was repeated at the end of each hour until a theoretical yield of holocellulose was obtained (estimated by subtracting from 100 the sum of the ash-free lignin, protein, and ash contents of the extracted leaves). The suspension was filtered by suction through a sintered glass crucible (C porosity), washed repeatedly with water and finally with acetone. The holocellulose residue was dried at room temperature. The yield of holocellulose was calculated on a moisture-, lignin-, ash-, and protein-free basis.

A series of one to eight treatments were carried out on beech, birch, maple and poplar leaves, which were partially decomposed after 11 months' weathering in trays (12). The yields of holocellulose and the lignin, protein, and ash contents of the crude holocellulose fractions obtained on successive treatment of beech leaves with acid chlorite are shown in Figure 1. Similar results were obtained with birch, maple and poplar leaves.

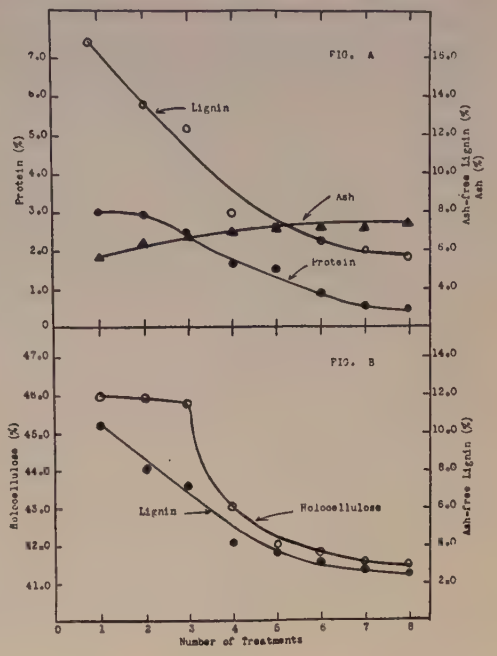


FIGURE 1. Effect of successive acid chlorite treatments on extractive-free beech leaves. (Exposed 11 months prior to analysis).

A. Ash, protein and ash-free lignin contents of crude holocellulose isolated after each treatment. (Basis: oven-dry crude holocellulose).

B. Yield of holocellulose and lignin content of the isolated fractions. (Basis: oven-dry extracted leaves).

Three treatments of beech, birch and maple leaves with acid chlorite gave theoretical yields of holocellulose. Poplar leaves required only two treatments (see Table 1). Longer digestion caused loss of holocellulose in all cases. The percentage of ash in the holocellulose fractions increased with each successive acid chlorite treatment. However, when calculated on the basis of extractive-free leaves, an actual decrease in ash occurred in all instances. Straw holocelluloses were found to contain more ash than the original extracted straw (2).

Delignification with sodium chlorite to obtain theoretical yields of holocellulose brought into solution from 74.4 to 84.0 per cent of the protein and from 77.2 to 87.1 per cent of the lignin on the basis of the oven-dry, extractive-free residues. The decrease in ash varied from 20.4 to 46.4 per cent. These results are shown in Table 1.

From the data given in Figure 1, it is evident that removal of lignin by successive acid chlorite treatments proceeded quite rapidly until the fourth treatment; the remaining lignin was tenaciously held by the cellulosic material and brought into solution only very slowly by further treatments. One chlorite treatment of beech leaves reduced the lignin from 38.54 to 10.51 per cent, based on the oven-dry extracted leaves. After four treatments the apparent lignin content was 4.14 per cent and four hours' further digestion decreased this only to 2.72 per cent. Complete removal of material reacting like lignin was not attained, even on prolonged treatment. Reference to Figure 1B shows that delignification treatments beyond three gave less than the theoretical yield of beech holocellulose and caused a progressive reduction in the carbohydrate material. Other workers report similar results in the preparation of holocellulose from cereal straws (2) and from wood (16, 44). The resistance of a part of the lignin to the action of

TABLE 1.—YIELDS OF HOLOCELLULOSE* AND REMOVAL OF LIGNIN, PROTEIN AND ASH ON DELIGNIFICATION OF EXTRACTED LEAVES WITH ACID CHLORITE

(Basis: 100 gm. oven-dry extracted leaves)

Species	Beech	Birch	Maple	Poplar
Number of chlorite treatments	3	3	3	2
Theoretical holocellulose content	45.5	27.4	43.4	35.5
Experimental yield of holocellulose	45.8	27.3	43.4	35.5
Percentage removal of lignin (ash-free)	81.2	87.1	77.2	83.4
Percentage removal of protein	74.4	80.1	84.0	76.6
Percentage removal of ash	26.0	46.4	20.4	42.1

* Corrected for content of ash, protein and ash-free lignin.

sodium chlorite may be due to the intimate association of lignin with cellulose; there is considerable evidence in support of a chemical union between lignin and carbohydrates in plant tissues (22, 26). Further, the effectiveness of sodium chlorite as an oxidizing agent in weak acid solutions decreases as the pH increases (38). In the experiments mentioned above, the pH of the suspensions increased in every case from about 4.2 to 5.0 during the first four treatments with chlorite, rising to 6.0 at the end of eight additions of the reagents. A similar increase in pH was noted by Adams and Castagne (2). Less effective delignification could be expected as the acidity decreased.

The results obtained on small samples indicated a fraction could be prepared containing most of the original carbohydrates in the plant tissue, freed from a large part of the lignin and protein. Such a fraction is particularly suited for research on the hemicelluloses which were of major interest. Therefore large lots of holocellulose (50 gm.) from mature leaves and residues were prepared by the method outlined for small samples, except that the suspension was stirred mechanically during the delignification step and the residual holocellulose was recovered and washed in a Buchner funnel lined with fine filter cloth. Proportionate amounts of reagents were added at hourly intervals and the temperature kept constant at 75 $\pm$ 0.5° C. Four chlorite treatments were employed, regardless of the species, in order to obtain fractions of low lignin content without prohibitive degradation of carbohydrate.

Analysis of Holocellulose from Freshly-Fallen and Partially Decomposed Leaves

Holocellulose fractions, prepared from freshly-fallen and from partially decomposed leaves by four successive treatments with acid chlorite, were analysed for ash, protein, ash-free lignin, furfural, uronic acid anhydride, acetyl and alpha-cellulose contents. The extracted leaves were analysed for the same constituents and the results are presented in Table 2. In order to facilitate comparison the data are presented on the basis of the moisture- and extractive-free leaf material.

It will be noted that four chlorite treatments resulted in appreciable losses of carbohydrates, except in fresh beech leaves. The loss of furfural from 1948 beech leaves was 2.9 per cent, while the decreases in uronic

acid anhydride and acetyl contents were 20.1 and 25.3 per cent, respectively. In the other species of leaves collected in 1948, losses of these constituents ranged from 19.5 to 47.1 per cent. With the exception of birch leaves, the carbohydrate fraction in the partially decomposed material was more easily degraded than that present in the fresh leaves. Approximately 85 to 90 per cent of the protein and 80 to 85 per cent of the lignin in the extracted leaves were removed by four chlorite treatments. Removal

TABLE 2.—ANALYSIS OF LEAVES AND THEIR HOLOCELLULOSE FRACTIONS OBTAINED BY FOUR CHLORITE TREATMENTS

(Basis: Moisture- and extractive-free leaves and leaf residues)

Analyses (per cent)	Materials	Beech	Birch	Maple	Poplar
Unweathered leaves collected in 1947					
Experimental yield	Holocellulose	50.08	41.27	47.99	46.37
Theoretical yield	Holocellulose	49.79	46.72	52.81	50.86
Deviation of exptl. yield from theoretical	Holocellulose	+0.58	−11.66	−9.12	−8.82
Ash	Leaves	6.20	4.39	8.39	4.17
	Holocellulose	6.34	4.52	8.18	6.13
Protein (N × 6.25)	Leaves	5.49	8.56	6.92	5.83
	Holocellulose	0.74	0.90	1.00	0.72
Lignin, ash-free	Leaves	34.23	36.26	27.71	34.22
	Holocellulose	5.76	8.80	7.91	9.02
Alpha-cellulose	Holocellulose	—	—	32.31	32.37
1947 leaves exposed 11 months in trays					
Experimental yield	Holocellulose	42.78	25.53	37.50	29.38
Theoretical yield	Holocellulose	45.49	27.36	43.39	35.51
Deviation of exptl. yield from theoretical	Holocellulose	−5.95	−6.68	−13.57	−17.23
Ash	Leaves	5.30	7.11	12.05	6.44
	Holocellulose	4.49	4.57	9.57	4.46
Protein (N × 6.25)	Leaves	5.80	12.19	7.02	6.54
	Holocellulose	0.87	1.12	1.02	0.81
Lignin, ash-free	Leaves	38.54	49.91	33.04	46.87
	Holocellulose	6.41	6.81	6.63	7.96
Alpha-cellulose	Holocellulose	—	—	21.79	15.79
Unweathered leaves collected in 1948					
Experimental yield	Holocellulose	50.86	39.37	51.23	43.78
Theoretical yield	Holocellulose	51.12	42.25	53.89	48.12
Deviation of exptl. yield from theoretical	Holocellulose	−0.51	−6.81	−4.93	−9.01
Ash	Leaves	3.41	5.79	9.90	5.08
	Holocellulose	3.27	4.67	8.54	4.63
Protein (N × 6.25)	Leaves	5.92	11.12	5.48	4.85
	Holocellulose	0.78	0.88	0.52	0.42
Lignin, ash-free	Leaves	34.37	36.36	24.02	36.77
	Holocellulose	4.23	7.83	4.56	6.77
Furfural	Leaves	12.53	9.76	10.20	11.17
	Holocellulose	12.17	6.32	6.66	9.01
Uronic acid anhydride	Leaves	10.88	12.48	15.50	11.20
	Holocellulose	8.69	6.60	11.02	7.03
Acetyl	Leaves	3.98	2.05	2.64	2.84
	Holocellulose	2.97	1.46	1.84	1.98
Alpha-cellulose	Holocellulose	25.00	21.96	32.81	25.81

of ash was variable, though somewhat greater from the exposed leaves than from the unweathered leaves. The high ash contents of the holocelluloses prepared from 1947 beech, birch and poplar leaves are probably due to insufficient washing of these fractions with distilled water.

Characteristics of Leaf and Holocellulose Lignin

The nitrogen and methoxyl contents of lignin isolated from unweathered and partially decomposed leaves, and from the holocellulose fractions of these materials, are given in Table 3.

It will be noted that leaf lignin contains appreciable amounts of nitrogen. Lignin from freshly-fallen birch and maple leaves contains more nitrogen than that from beech and poplar. In general, from one-third to one-half of the nitrogen present in the leaf tissue is associated with the isolated lignin. Since treatment with chlorite is effective in removing a large portion of the nitrogen it is not surprising that holocellulose "lignin" contains relatively little nitrogen.

The methoxyl content of leaf lignin is low compared to that of lignin isolated from woods (15). The values are of the same magnitude as reported for lignin isolated from succulent plant tissues (20), soil (10), pasture herbage and feces of sheep (35). Beech leaf lignin has the highest methoxyl content, followed by maple, poplar and birch lignins. The data indicate that weathering has little effect on the composition of the leaf lignin.

TABLE 3.—THE NITROGEN AND METHOXYL CONTENTS OF THE KLASON LIGNIN ISOLATED FROM LEAVES, LEAF RESIDUES AND THEIR HOLOCELLULOSE FRACTIONS

(Basis: 100 gm. of moisture- and ash-free lignin)

Species	Lignin from leaves		"Lignin" from holocellulosee	
	N	OCH_3	N	OCH_3
Unweathered leaves collected in 1947				
Beech	1.32	6.75	0.44	2.28
Birch	1.96	3.81	0.29	1.13
Maple	2.08	5.20	0.51	3.80
Poplar	1.39	4.53	0.32	1.92
1947 leaves exposed in trays for 11 months				
Beech	1.55	7.16	0.31	2.64
Birch	2.06	4.25	0.33	1.02
Maple	1.65	6.78	0.40	2.07
Poplar	1.70	4.98	0.28	1.09
Unweathered leaves collected in 1948				
Beech	1.59	7.30	0.52	2.34
Birch	1.82	3.94	0.30	1.43
Maple	1.69	6.55	0.34	1.83
Poplar	1.14	4.91	0.18	0.48

"Lignin" isolated from holocellulose has a much reduced methoxyl content compared to that of lignin from the corresponding extractive-free tissue. Acid chlorite digestion either demethoxylates the lignin or removes methoxyl-containing interfering substances, or the material in holocellulose which is insoluble in 72 per cent sulphuric acid is quite different chemically from the acid-insoluble material in the original tissues. There is some basis for the latter suggestion. Holocellulose lignin was tough, elastic and nearly colourless compared to the dark chocolate brown, friable material isolated from the original tissue. So-called "lignin" isolated from leaf holocellulose may be carbohydrate in nature as it exhibits physical characteristics suggestive of plant gums or mucilages.

Determination of Sugars in Acid Hydrolysates of Leaf Holocelluloses

The holocellulose fractions prepared by four acid chlorite treatments of beech, birch, maple and poplar leaves (unweathered 1948 collection) were hydrolyzed with dilute sulphuric acid and the hydrolysates examined for sugars by the paper partition chromatographic technique.

In a typical experiment the holocellulose (0.5 gm.) was hydrolyzed by heating with 1 N sulphuric acid (12.5 ml.) in a sealed tube on a boiling water bath for 12 hours. The suspension was centrifuged and the insoluble residue washed with a small quantity of hot water. The filtrate and washings were combined and neutralized with solid barium carbonate using Congo red as the indicator. The barium sulphate was removed by centrifuging, washed with two to three millilitres of distilled water and the washings added to the filtrate which was concentrated to a syrup at room temperature. The water-soluble barium salts were precipitated by the addition of a large excess of ethanol and removed by centrifuging. The alcoholic filtrate was concentrated as above. The syrup was dissolved in two millilitres of distilled water and the solution examined for sugars as described in the following section.

The sugars were detected by descending paper chromatography as outlined by Partridge (25). The solvent system of ethyl acetate-acetic acid-water as recommended by Jermyn and Isherwood (17) was found suitable for the separation of xylose, glucose and galactose. Arabinose could not be positively identified with this solvent since mannose was found to have almost the same R_f value. The chromatograms were developed in the above solvent system for 24 and 36 hours. The longer period gave more complete separation of glucose and galactose. The presence of arabinose in the hydrolysates was ascertained by developing the chromatograms with phenol saturated with water. In this solvent arabinose moves faster than mannose and hence can be easily separated from it. The developed chromatograms were dried in an electric oven at 105° C. and the sugar spots made visible using the procedure outlined by Trevelyan, Procter and Harrison (39).

The effect of varying the concentration of sulphuric acid and the time of hydrolysis is well illustrated by the chromatograms shown in Figure 2. Hydrolysis of beech holocellulose with 0.2 N acid for one hour splitoff arabinose and small amounts of galactose and xylose. No glucose was present in the hydrolysate. Very faint spots of slow-moving substances are discernible; these are presumed to be di-, tri-, or higher polysaccharide

fragments. Increasing the acid concentration to 1 N resulted in further hydrolysis of the holocellulose. Xylose, arabinose and galactose show well defined spots, the spots presumed due to polysaccharide fragments are more pronounced and the presence of glucose in the hydrolysate is indicated. On lengthening the time of hydrolysis to four hours the spots indicating incompletely hydrolysed fragments disappeared. Beech holocellulose appears to be completely hydrolysed in four hours as no more sugars were detected when the hydrolysis was continued for 12 hours. On the basis of these results all further hydrolyses were carried on for 12 hours using 1 N sulphuric acid.

Xylose, arabinose, glucose and galactose were detected in each of the holocellulose fractions examined. Figure 3 is a photograph of the original chromatograms of maple hydrolysate, developed as described above. A mixture of known sugars are spotted on each chromatogram for purposes of comparison. Similar results were obtained with hydrolysates of beech, birch and poplar holocellulose.

Xylose, arabinose and galactose appear to be relatively common constituents of plant hemicelluloses (3, 4, 6, 7, 8, 9, 21, 23, 42), whereas glucose is seldom encountered. Recently glucose was specifically identified as a constituent of wheat straw hemicellulose (6). In the present study, glucose may have resulted from hydrolysis of alpha-cellulose although this would not be expected to occur to any extent in a dilute acid solution. More detailed investigation of the holocellulose fractions is desirable.

SUMMARY

Chlorite holocellulose fractions of extractive-free hardwood leaves apparently retained 13 to 23 per cent of the original lignin and from 16 to 26 per cent of the original protein present in the tissues.

Beech, birch and maple leaves required three treatments and poplar leaves two treatments with acid chlorite to obtain a theoretical yield of holocellulose.

A relatively large proportion of the lignin in extracted leaves is easily brought into solution by the reagent while a small amount appears to be tenaciously held and not removed by chlorite without simultaneous degradation of the carbohydrate substances.

With the exception of that from birch, the carbohydrate fractions in partially decomposed leaves were more readily attacked by chlorite than those present in unweathered leaves.

Hardwood leaf lignin contains appreciable amounts of nitrogen; from one-third to one-half of the nitrogen present in the leaf tissue is associated with the isolated lignin.

The methoxyl contents of the various leaf lignins ranged from 3.81 to 7.30 per cent, being highest in beech followed by maple, poplar and birch. Lignin isolated from partially decomposed leaves (1947 collection) contained more methoxyl than lignin from the corresponding unweathered leaves.

Lignin isolated from holocellulose had a much reduced nitrogen and methoxyl content, compared to that of lignin from the corresponding extractive-free tissue.

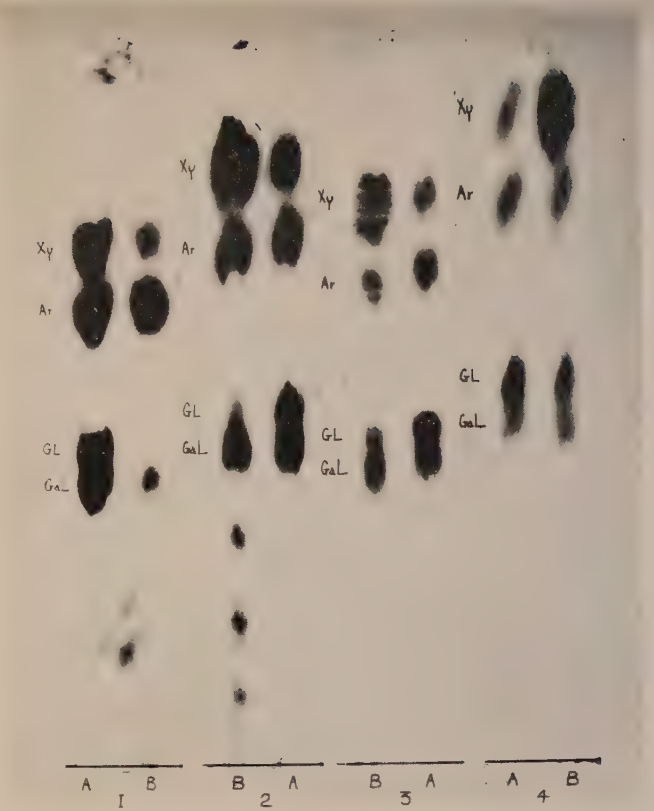


FIGURE 2. Hydrolysis of beech holocellulose for different periods.
A = known sugars; B = hydrolysate; X_y = xylose;
Ar = arabinose; Gl = glucose; Gal = galactose.
No. 1—0.2 NH_2SO_4; time—1 hr.
No. 2—1.0 NH_2SO_4; time—1 hr.
No. 3—1 NH_2SO_4; time—4 hr.
No. 4—1 NH_2SO_4; time—12 hr.

FIGURE 3. Chromatograms of hydrolysate from maple holocellulose.
A = known sugars; B = hydrolysate;
Xy = xylose; Ar = arabinose;
Gl = Glucose; Gal = galactose.
No. 10—Time of run 24 hr.; solvent—
EtAc + HAc + H_2O
No. 11—Time of run 36 hr.; solvent—
EtAc + HAc + H_2O
No. 12—Time of run 36 hr.; solvent—
Phenol.

Lignin isolated from leaf holocellulose was tough, elastic, and nearly colourless, compared to the dark chocolate brown, friable material obtained from the original tissue, which suggests that the two lignins differ in chemical composition.

Xylose, arabinose, glucose and galactose were identified by paper chromatography, in the acid hydrolysates of beech, birch, maple and poplar leaf holocellulose.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude their indebtedness to officers of the Experimental Farms Service, Canada Department of Agriculture, whose advice, support and assistance made this investigation possible.

REFERENCES

1. Adams, G. A., and A. E. Castagne. Some factors affecting the determination of furfural. Can. J. Research, *B*, 26 : 314-324. 1948.
2. Adams, G. A., and A. E. Castagne. Holocelluloses from straws. Canadian J. Research, *B*, 26 : 325-334. 1948.
3. Anderson, E., and P. W. Krznarich. Hemicellulose from oat hulls. J. Biol. Chem, 111 : 549-552. 1935.
4. Anderson, E., and H. J. Lowe. The composition of flaxseed mucilage. J. Biol. Chem. 168 : 289-297. 1947.
5. Bennett, E. Preparation of holocellulose from non-woody plant material. Anal. Chem. 19 : 215. 1947.
6. Bishop, C. T., and G. A. Adams. Use of liquid ammonia in the fractionation of wheat straw holocellulose. Can. J. Research, *B*, 28 : 753-761. 1950.
7. Buston, H. W. The polyuronide constituents of forage grasses. Biochem. J. 28 : 1028-1037. 1934.
8. Buston, H. W. Observations on the nature, distribution and development of certain cell-wall constituents of plants. Biochem. J. 29 : 196-218. 1935.
9. Buston, H. W., and V. H. Chambers. Some cell-wall constituents of *Cetraria islandica* ("Iceland Moss"). Biochem. J. 27 : 1691-1701. 1933.
10. Cann, E. D. Fractionation of soil organic matter. *Doctoral Dissertation*, McGill University. 1946.
11. Clark, E. P. The Vieböck and Schwappach method for the determination of methoxyl and ethoxyl groups. J. Assoc. Official Agr. Chem. 15 : 136-140. 1932.
12. Coldwell, B. B., and W. A. DeLong. Studies of the composition of deciduous forest tree leaves before and after partial decomposition. Sci. Agr. 30 : 456-466. 1950.
13. Dickson, A. D., H. Otterson, and K. P. Link. A method for the determination of uronic acids. J. Amer. Chem. Soc. 52 : 775-779. 1930.
14. Genung, L. B., and R. C. Mallatt. Analysis of cellulose derivatives. Ind. Eng. Chem., *Anal Ed.* 13 : 369-374. 1941.
15. Harris, E. E. Some characteristics of wood lignins. J. Amer. Chem. Soc. 58 : 894-897. 1936.
16. Jayme, G. Preparation of holocellulose and cellulose with sodium chlorite. Cellulosechemie, 20 : 43-49. 1942. *Abstracted in* Chem. Abstracts 37 : 766. 1943.
17. Jermyn, M. A., and F. A. Isherwood. Improved separation of sugars on the paper partition chromatogram. Biochem. J. 44 : 402-407. 1949.
18. Lemieux, R. U. "Hindrance effects" in cellulose substitution reactions. *Doctoral Dissertation*, McGill University. 1946.
19. Lovell, E. L. Fibrous holocellulose from softwoods. Ind. Eng. Chem. 37 : 1034-1037. 1945.
20. MacDougall, D., and W. A. DeLong. Effect of initial drying temperature on the apparent lignin content of plant tissues. Can. J. Research, *B*, 20 : 40-48. 1942.
21. McIlroy, R. T., G. S. Holmes, and R. P. Mauger. A preliminary study of the polyuronide hemicellulose of *Phormium tenax* (N.Z. flax). J. Chem. Soc. (London), pp. 796-799, 1945.
22. Norman, A. G. *In* Cellulose and cellulose derivatives, pp. 427-446, ed. by Emil Ott. Interscience Publishers, Inc., New York, N.Y. 1943.
23. Norris, F. W., and I. A. Preece. Studies on hemicelluloses. I. The hemicelluloses of wheat bran. Biochem. J. 24 : 59-66. 1930.

REFERENCES—Continued

24. Official and tentative methods of analysis, 6th ed. Assoc. of Official Agr. Chemists. Washington, D.C. 1945.
25. Partridge, S. M. Application of the paper partition chromatogram to the qualitative analysis of reducing sugars. Nature 158 : 270-271. 1946.
26. Purves, C. B. *In* Cellulose and cellulose derivatives, pp. 101-106, ed. by Emil Ott. Interscience Publishers, Inc., New York, N.Y. 1943.
27. Ritter, G. J. Determination of alpha-cellulose. Ind. Eng. Chem., *Anal. Ed.* 1 : 52-54. 1929.
28. Ritter, G. R., R. M. Seborg, and R. L. Mitchell. Factors affecting quantitative determination of lignin by 72 per cent sulfuric acid method. Ind. Eng. Chem., *Anal. Ed.* 4 : 202-204. 1932.
29. Samsel, E. P., and J. A. McHard. Determination of alkoxyl groups in cellulose ethers. Ind. Eng. Chem., *Anal. Ed.* 14 : 750-754. 1942.
30. Schmidt, E., and E. Graumann. Incrusting substances of plants. I. Methods for the preparation of pure plant skeletal substances. Ber. B, 54 : 1860-1873. 1921. *Abstracted in* Chem. Abstracts 16 : 273. 1922.
31. Schmidt, E., K. Meinel, W. Jandebeur, and W. Simson. Die Kettelänge der Cellulosen Nativer Zusammensetzung und die Kettenlänge des Acetyl-Xylans der Laubhölzer. Cellulosechemie 13 : 129-139. 1932.
32. Schmidt, E., K. Meinel, and E. Zinth. Plant membranes. Ber., B, 60 : 503-526. 1927. *Abstracted in* Chem. Abstracts 21 : 1829. 1927.
33. Schmidt, E., Y. Tang, and W. Jandebeur. Preparation of skeleton substances from encrusted cell-walls by chlorine dioxide in the "one step procedure". Cellulosechemie 12 : 201-212. 1931. *Abstracted in* Chem. Abstracts 26 : 1772. 1932.
34. Sohn, A. W., and F. Reiff. Sodium chlorite as a pulping agent. Papier-Fabr. 40 : 1-7 : 105-112. 1942. *Abstracted in* Chem. Abstracts 37 : 1865. 1943.
35. Sowden, J. F. Studies on lignin and related compounds in forage and in animal excreta. *Doctoral Dissertation*, McGill University. 1944.
36. Stillings, R. A., and B. L. Browning. Photocolorimetric determination of furfural. Ind. Eng. Chem., *Anal. Ed.* 12 : 499-502. 1940.
37. TAPPI official standard T 9 M-45. Sept., 1945.
38. Taylor, M. C., J. F. White, G. P. Vincent, and G. L. Cunningham. Sodium chlorite. Ind. Eng. Chem. 32 : 899-903. 1940.
39. Trevelyan, W. E., D. P. Procter, and J. S. Harrison. Detection of sugars on paper chromatograms. Nature 166 : 444-445. 1950.
40. Van Beckum, W. G., and G. J. Ritter. Rapid methods for the determination of holocellulose and Cross and Bevan cellulose in wood. Paper Trade J. 105 : No. 18, pp. 127-130. 1937.
41. Van Beckum, W. G., and G. J. Ritter. Chemical composition of wood. Paper Trade J. 104 : No. 19, pp. 49-50. 1937.
42. Weihe, H. D., and M. Phillips. Hemicelluloses of wheat straw. J. Agr. Research 60 : 781-786. 1940.
43. Wise, L. E. Quantitative isolation of hemicelluloses from coniferous woods. Ind. Eng. Chem., *Anal. Ed.* 17 : 63-64. 1945.
44. Wise, L. E., M. Murphy, and A. A. D'Addieco. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. Paper Trade J. 122 : No. 2, pp. 35-43. 1946.

AIR PURIFICATION AND SOLUBLE PECTIN ACCUMULATIONS IN LAWFAM APPLES[1]

P. A. Poapst and W. R. Phillips

Division of Horticulture, Central Experimental Farm, Ottawa

[Received for publication April 12, 1951]

Considerable controversy has developed in recent years over the employment of activated charcoal beds as a deterrent to ripening apples in cold store. Recent researches (4) point to the possibility of ripening type emanations from apples other than ethylene which may be adsorbed on carbon. If this is so, the picture concerning adsorption will probably remain shrouded in mystery until these apple volatiles are identified and their significance ascertained. However, it is believed that this difficulty will be overcome and a suitable selective adsorbent will be eventually devised. In the meantime, it was felt that some information regarding the "optimum" effect which could be secured by good adsorption would be useful knowledge. The word "optimum" is used with some reservation, as it will be observed later that the method used to eradicate the volatiles would never accomplish this completely and no volatile measurements were attempted.

The selection of a working material and the anticipation of what results might be expected was assisted somewhat by other studies. Previous scald control investigations, employing activated and brominated charcoals as volatile filters, suggested that increments gained in storage life would be slight and difficult to assess accurately. At the time of the scald control studies it had been noticed that comparative ballast material of the Lawfam variety had responded favourably to the filtered environment, to the extent of a two-pound pressure difference at the end of storage. Published evidence (5) suggests that there is a varying varietal response to activated charcoal filters for unknown reasons. In this respect early maturities (pre-climacteric) respond more favourably (3). For the above reasons, an early pick of the Lawfam variety was selected as the experimental material.

The significance (1) of the degradation of the protopectin or insoluble pectin constituents of the apple has been shown to be intimately associated with the ripening phase. Usually the protopectins are converted into soluble products by enzyme action or otherwise, with a resultant loosening and softening of the tissues, which accompanies maturation. The accumulation of the soluble pectins is a transitory or intermediate phase to the development of lesser products; but the trends serve as useful data in estimating the condition of a ripening apple. Periodic evaluations, to determine the state of an apple in storage during experiments involving controlled atmospheres, fertilizer applications, variation of temperatures and picking maturity, have proved this measurement to be a sensitive and useful criterion. It was found generally that longer storing apples followed lower levels of soluble pectin accumulation. The procedure (2) outlined by Carré and Haynes was adopted for this work, performed on the clarified expressed juices of 10 apple samples.

[1] Contribution No. 755 from the Division of Horticulture, Experimental Farms Service.

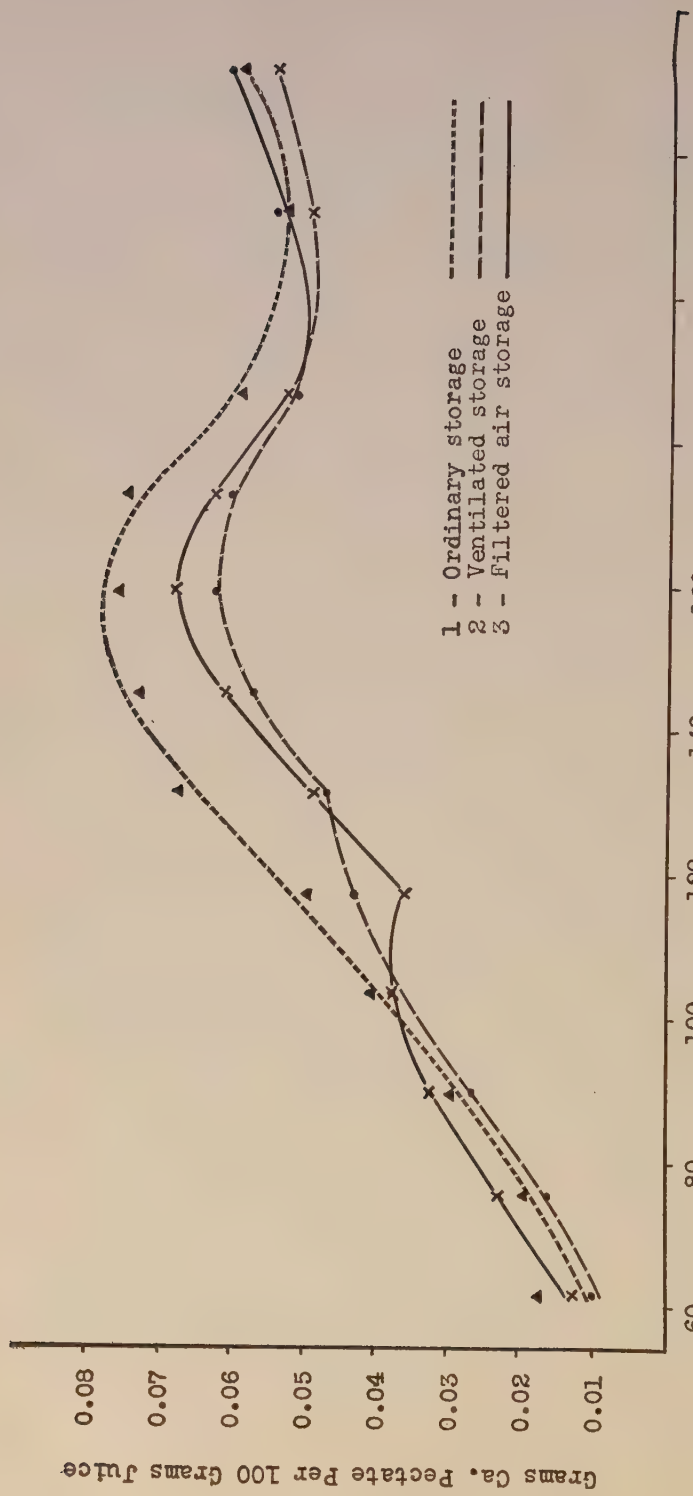


FIGURE 1. Soluble pectin trends in early maturity Lawfam apples held in (i) ordinary storage (ii) ventilated storage (iii) filtered air storage.

Fixed equipment for this test consisted of three test chambers of similar shape and design. Each chamber was of 1,000 cubic feet approximate capacity and fitted with overhead grid coils for cooling. The temperature was controlled at 32 ± 1° F.

In order to ensure a positive removal of volatiles, one chamber was vented to a source of fresh air. A furnace blower provided an input of 50 c.f.m. Thermostatic regulation stopped the fan operation when room temperature reached 31° F. However, heat leakage was such that the fan operated almost continuously. On the high temperature side, cooling was provided by the overhead coils.

An activated carbon adsorption assembly of commercial manufacture was installed in another chamber. The unit housed four cannisters, containing a total of six pounds of activated coconut shell carbon and was provided with an oiled, honeycombed paper air straightener and a propellor type fan. Air circulation was regulated at 75 c.f.m. for half the storage period and then dropped to 45 c.f.m. Manufacturer's specifications for this model listed 1200 bushels as design capacity.

All three chambers were similarly loaded with several varieties of apples to the extent of 300 bushels per unit. The test material (secured especially for its uniformity) was distributed among the three rooms. The carbon cannister unit and the ventilation assembly were turned on after 58 days of storage; by this time it was presumed that normal physiological trends would have established themselves. A later pick of Lawfam was distributed between the check or control chamber and the ventilated chamber.

Figures 1 and 2 show the plot of percentage pectin accumulation as its calcium salt referred to days in storage. Quality (flavour and texture) evaluations, as determined by three operators, found eating ripeness to coincide with peak accumulations at approximately 160 and 150 days of storage. It is noted in both figures that comparative curves assumed peak values coincidentally, and presumably samples reached optimum eating condition on the same time interval. The suppression of the pectin accumulation demonstrated by the filtered and ventilated samples suggests a greater retention of quality after optimum ripeness was obtained. This has been found to be the case as in previously mentioned work on gas storage, etc. It was estimated in the present experiment from quality records that up to 18 days' additional storage life were gained by the carbon filtration. In the case of the ventilated samples, shrinkage had made such inroads by roughly 200 days of storage that real comparison could not be made. The indentation in the second trend (Figure 1), with a resumption of the former slope, is curious. It suggests adsorption and desorption or saturation. Unfortunately there are no data to substantiate this.

Examination for physiological or storage disorders revealed nothing of significance; except for shrinkage the fruit remained visibly sound throughout the test. No great divergence was experienced in the decline of total titratable acidities. There was a suggestion of gentler slope in the ventilated samples, but this was outside the experimental accuracy. Soluble solids remained reasonably constant throughout the storage interval with a tendency to taper off to a slightly lower level in the check samples only.

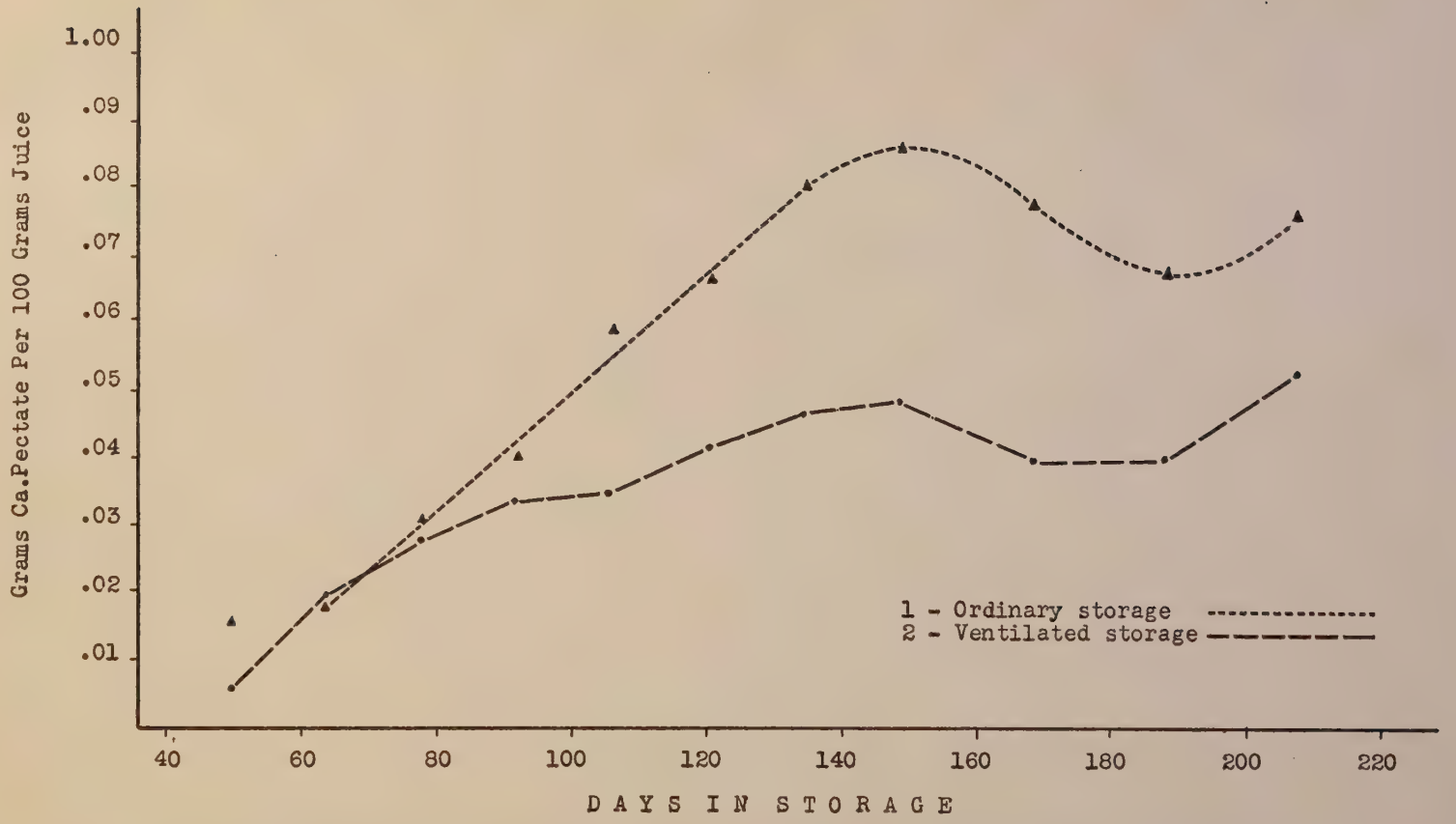


FIGURE 2. Soluble pectin trends in later maturity Lawfam apples held in (i) ordinary storage (ii) ventilated storage.

It is realized that the study of volatiles and their adsorption or removal is a highly controversial field; however, it is felt that the results of the pectin plots are striking and that they may shed some light on what effects air purification may have on fruit metabolism.

REFERENCES

1. Carré, M. H. An investigation of the changes which occur in the pectin constituents of stored fruit. Biochem. J. 16 : 704-712. 1922.
2. Carré, M. H., and D. Haynes. The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble pectin in apples. Biochem. J. 16 : 60-69. 1922.
3. Smock, R. M. The influence of stored apples on the ripening of other apples stored with them. Cornell Univ. Agr. Exp. Sta. Bull. 799, pp. 1-36. 1943.
4. Smock, R. M., and C. R. Gross. Studies on respiration of apples. Cornell Univ. Agr. Exp. Sta. Memoir 297, pp. 38-40. 1950.
5. Smock, R. M., and F. W. Southwick. Air purification in the apple storage. Cornell Univ. Agr. Exp. Sta. Bull. 843, pp. 1-52. 1948.

INFECTION OF BASAL SHOOTS OF HOP PLANTS BY *RHIZOCTONIA SOLANI*[1]

By Walter Jones[2]

Dominion Laboratory of Plant Pathology, Saanichton, B.C.

[Received for publication December 14, 1951]

During May, 1950, an unrecorded disease of hop plants, caused by the infection of young basal shoots by the fungus *Rhizoctonia solani* Kühn was observed in a 50-acre hop yard of the Brewer's Gold variety in British Columbia.

A routine operation of hop growers in early spring is the removal of the soil from around the hills and the cutting back of the shoots (straps) close to the crowns of the root-stock. Following this, some of the growers cover the crowns with soil. The vines that develop later are the ones that are trained on strings.

In this particular hop yard, the crowns had been covered with soil after the cutting operation. The young shoots that developed later below the soil were thus not exposed to daylight and were whitish in colour. It was at this stage that the infection of the young shoots by *R. solani* occurred in the field.

Brown necrotic lesions characteristic of Rhizoctonia infection were prevalent on infected shoots at different points, and often these lesions were present on the terminal and lateral buds, as well as on the basal areas of the shoots close to the crown. Mycelium of *R. solani* was present on and within the tissues of these lesions.

Growth was thus retarded and many of the shoots were killed (Figure 1). Where infection had taken place at the base of a shoot, the affected shoot was usually easily detached from the crown at that point. In general, the symptoms were similar to those associated with Rhizoctonia infection of sprouts and stolons of potato plants.

The fungus was isolated from the necrotic tissues, and its pathogenicity was established by inoculating growing shoots of hop plants of the Golding variety in the greenhouse.

Small crowns of the Golding variety were planted in autoclaved soil and covered with inverted earthenware pots to exclude any light. The shoots that developed later were whitish in colour, similar to those in the field where covered with soil. At this stage, inoculum of the fungus grown on corn meal plus vermiculite was placed on the growing shoots at different points. Following this, the inoculated and check plants were covered again with the inverted pots to exclude light. In approximately a week, definite symptoms of Rhizoctonia infection became evident on the inoculated shoots (Figure 1).

In another series, the crowns were left uncovered, and the shoots that developed were greenish in colour. These shoots were also inoculated but no infection occurred.

---

[1] Contribution No. 1130 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

[2] Associate Plant Pathologist. Dominion Laboratory of Plant Pathology, Saanichton, B.C.

FIGURE 1.—Rhizoctonia infection of the basal shoots of a hop plant. *Left*—Control plant, not inoculated. *Right*—Diseased shoots; the one on the left has collapsed due to infection at its base.

To avoid a recurrence of the disease, the grower was advised to leave the crowns exposed after the cutting operation.

During the 1951 season, the grower did not cover the crowns with soil after the cutting operation and as a result there was no evidence of field infection, as judged by uniformity of stand.